SELECTION AND CHARACTERIZATION OF RESISTANCE IN MANGO (*Mangifera indica* L.) EMBRYOGENIC CULTURES TO THE PHYTOTOXIN PRODUCED BY *COLLETOTRICHUM GLOEOSPORIOIDES* Penz.

By

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To
My beloved parents,
Mrs. and Mr. R. Subramanian

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by

Jayasankar Subramanian

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Abstract of Dissertation Presented to the Graduate School
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By

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Major Department: Horticultural Sciences

Mango (*Mangifera indica* L.) is the most important fruit crop of Asia and currently ranks fifth in world production. Anthracnose, a necrotic disease caused by the fungus, *Colletotrichum gloeosporioides* Penz., is the major production and post-harvest problem of all mango growing regions of the world. Introduction of anthracnose resistance into existing mango cultivars by conventional breeding has been unsuccessful. Moreover, control of anthracnose by fungicides has become increasingly environmentally unsustainable. Together with other somatic cell genetic approaches, *in vitro* selection can address certain specific plant breeding goals in perennial fruit breeding. The current study has involved the establishment of resistance to *C. gloeosporioides* in two mango cultivars, 'Hindi' and 'Carabao,' by recurrently selecting

embryogenic suspension cultures with fungal culture filtrate or a partially purified phytotoxic metabolite and the characterization of these lines at the subcellular level. The culture filtrate, obtained by growing a virulent strain of *C. gloeosporioides,* was partially purified. Using standard bioassays, the phytotoxic metabolite was shown to have characteristics of phytotoxic activity. Embryogenic suspension cultures of the two mango cultivars were recurrently selected *in vitro* using the culture filtrate, or the phytotoxic metabolite and resistant lines were established. These resistant lines inhibited fungal mycelial growth in dual culture due to the production of high levels of extracellular antifungal proteins. Characterization of the extracellular proteins produced by the resistant lines revealed the presence of new isozymes of chitinase as well as enhanced secretion of other isozymes of chitinase and β-1,3-glucanase in comparison with the unchallenged controls. Both chitinase and β-1,3-glucanase are well known pathogenesis-related proteins involved in disease resistance. RAPD analysis of these near isogenic lines revealed variation in RAPD generated markers between unchallenged controls and the *in vitro* selected resistant lines of both cultivars. Unchallenged controls did not show variation from the leaf DNA of the respective parent trees, indicating the maternal origin of the cultures as well as their stability in suspension culture. Together, the results suggest that recurrent *in vitro* selection using culture filtrate or phytotoxic metabolite can be used for enhancing resistance to *C. gloeosporioides* in mango, which is probably conferred by the activation of certain disease-resistance genes existing in mango.

CHAPTER 1
INTRODUCTION

Mango is the major tropical fruit of Asia. Currently, it ranks fifth in production among all fruit crops worldwide after *Musa* (bananas and plantains), grapes, citrus and apples (FAO Production Yearbook, 1993). The total production in 1992 was estimated to be ca. 17.7 million metric tons. India produces approximately 60% of the world's mangos, most of which are consumed locally as fresh fruit. Mexico is the second largest producer, accounting for 6.4% of total production and is the biggest exporter of fresh mangos. Only 1% of the total production worldwide is utilized for processing. Mango fruit at all stages of development is consumed. Tender mangos are used for pickles and ripe mangos make delicious drinks, ice-cream, yoghurt and mango leather. Even the seeds are used for making "amchur," a dry starchy powder. There has been a sharp increase in mango exports in the past two decades and the export market is still expanding in N. America and Europe. It has been estimated that fresh mango imports increased by 120% in Europe and 53% in N. America between 1985 and 1990 (Galan, 1993). Nutritionally, mango fruits are a rich source of vitamins A and C and carbohydrates.

Like most tropical fruit species, mango is attacked by a number of pathogens. Anthracnose, caused by the fungal pathogen, *Colletotrichum gloeosporioides* Penz. is considered to be the most important production and postharvest problem of mango (Ploetz and Prakash, in press). Most of the commercial mango cultivars are highly susceptible to this disease.

Despite its economic importance, the genetics of mango are poorly understood (Iyer and Degani, in press). Although mango breeding for several important agronomic characters, including anthracnose resistance, is considered essential, conventional breeding has been mostly unsuccessful (Sauer, 1993), and has resulted in few cultivar releases. The highly seasonal nature of mango flowering offers a narrow opportunity for controlled pollination, and the frequency of premature fruit drop exceeds 99%.

Recently, unconventional breeding methods such as *in vitro* selection, protoplast fusion and genetic transformation have been considered to be essential supplements for breeding programs (Hammerschlag, 1992). Successful demonstration of *in vitro* selection for disease resistance has been reported in peach (*Prunus persica* L.) (Hammerschlag, 1988) and lemon (*Citrus limon* (L.) Burm.) (Gentile *et al.*, 1992a). The current study addresses the potential for using *in vitro* selection for enhancing anthracnose resistance in mango.

CHAPTER 2
REVIEW OF LITERATURE

2.1 Mango

2.1.1 Taxonomy

The mango, *Mangifera indica* L., is a member of the Anacardiaceae, a largely tropical group of plant species that is distributed both in the Old and New World. The Anacardiaceae also contains other species of economic importance such as the cashew, *Anacardium occidentale* L., pistachio, *Pistachio vera* L. and the mombins, *Spondias* spp. Bompard (1993) recognized 69 valid *Mangifera* species that were distributed from India and Sri Lanka to the Philippines. The genus is divided into two sections, *Mangifera* and *Limus,* on the basis of the presence or absence of floral disc (Bompard, 1993; Mukherjee, in press). Although edible fruits are produced by 14 *Mangifera* species, all of the commercial cultivars belong to *Mangifera indica* (Mukherjee, in press). The fruit of many wild species are poisonous. Wild *Mangifera* species are small with thin acidic flesh, large stones, abundant fiber and are often highly resinous.

2.1.2 Origin, Domestication and Distribution

Ancient records and accounts of travellers suggested an Indian origin of mango, from where it was believed to have spread outward to southeast Asia and the New World. The greatest species diversity of *Mangifera*, however, is found in the Malay peninsula (Mukherjee, 1985; Kostermanns and Bompard, 1993) and Borneo (Schnell and Knight, 1993). Recent molecular evidence suggests that *Mangifera* probably evolved in a large area that included northeast India and the Indonesian archipelago (Bompard and Schnell, 1996).

Mangifera probably evolved in a large area that included northeast India and the Indonesian archipelago (Bompard and Schnell, in press).

It is highly probable that the domestication of mango occurred in India. DeCandolle (1904) indicates that mango cultivation began at least 4,000 years ago in India. India exercised a strong cultural and religious influence at that time and domestication of mango in southeast Asia could have been the result of Buddhist monks and other travellers from India. Many commonly used words for mango in southeast Asia are derived either from Sanskrit or Tamil, e.g., the Malay name for mango is "maangga," which is derived from the Tamil "manga."

The mango has been a very important cultural and religious symbol of India. The Dravidians consider the mango to be highly sacred and mango leaves and fruits are used in religious functions of south India. Mention of mango groves in the times of Gautama Buddha (ca. 500 B.C.) has been made by the Chinese travellers, Fa-Hien and Yu-Sung (Popenoe, 1932). The stupa of Bharut, constructed ca.100 B.C., depicts a mango tree (Burns and Prayag, 1921). Other historical mentions of mango include those of the Chinese traveller, Hwen-T'sung (632-645 A.D.), the Arabs, Ebn Hankal (902-968 A.D.) and Ebn Batuta (1325-1349 A.D.) and the Portuguese, Lurdovei de Varthema (1503-1508 A.D.) (Mukherjee, in press). The introduction of vegetative propagation by the Portugese in the fifteenth century, a technique which was unknown in India until then (Iyer and Degani, in press), had an immediate impact on mango cultivation. The 'Lakh Bagh', near Darbanga in Bihar, a mango orchard of 100,000 trees, was planted by the Moghul emperor Akbar (1556-1605 A.D.) with approach grafted elite mango trees from various parts of India. This shows the importance and the esteem in which mango was held in India. Moreover, Ain-I-Akbari, the encyclopedic work compiled during the reign of Akbar describes

mango cultivars and cultivation in detail. It is believed that several important cultivars such as 'Dashehari,' 'Langra,' 'Alphonso,' 'Safdar Pasand,' 'Rani Pasand,' 'Jehangir' and 'Himayuddin' had their origin during that time and are still maintained by vegetative propagation (Mukherjee *et al.*, 1983).

Although mango has been cultivated in its original centers of domestication for a very long time, its outward spread did not occur until the beginning of Spanish and Portugese voyages of discovery in the fifteenth and sixteenth centuries. Purseglove (1969) suggested that mango might have been introduced into Africa via Persia and Arabia in the 10th century; however, it is probably unlikely, considering the recalcitrant nature of mango seeds. The Portugese spread mango from their Indian settlements to African colonies and thence to Brazil. The Spaniards introduced the mango from the Philippines to their New World colonies through the pacific ports of Acapulco in Mexico and Porto Bello in Panama (Mukherjee, in press). This view is further attested by the cultivars that are present today in Africa and Brazil (monoembryonic Indian types) and Mexico (e.g., 'Manila,' a polyembryonic cultivar from the Philippines).

2.1.3 Botany

The mango plant is an arborescent evergreen tree, with simple, alternate, oblong ovate to oblong lanceolate leaves. While the young leaves are tender and pinkish to copper colored, the mature leaves are tough, leathery and dark green in color. Flowers are borne in terminal panicles; the inflorescence is a widely branched, erect raceme. The flowers are small, monoecious and polygamous with both male and bisexual flowers occurring within the same inflorescence (Mukherjee, in press). Both sepals and petals are 4-5, ovate to oblong ovate and pubescent. Usually, the

color of the petals is creamy white, but it varies with cultivar. The disc is fleshy, large and located above the petals. There are 4-5 stamens, but only 1 or 2 are fertile; the remainder are sterile staminodes. The mango fruit is a large fleshy drupe. The edible mesocarp is resinous, fibrous and varies in thickness. While the unripe mango fruit is green, the fruit color at maturity is genotype-dependent and varies from green to yellow to blushes of pink or red.

The mango seed is large, flat, enclosed in a hard endocarp and is usually referred to as the 'stone.' The testa is thin and papery. Based on the seed type, mango is classified as either monoembryonic or polyembryonic. The cultivars of Indian origin, e.g., 'Mulgoa,' are monoembryonic with a single zygotic embryo. The cultivars of southeast Asian origin, e.g., 'Carabao,' are polyembryonic with one or more embryos; usually one embryo is zygotic and the remaining embryos are derived from the nucellus, the innermost integumental layer (Mathews and Litz, 1992; Iyer and Degani, in press). Occasionally a polyembryonic cultivar contains only one embryo, which may be zygotic or nucellar; however, certain polyembryonic cultivars such as 'Strawberry' (Juliano, 1934), 'Carabao' and 'Pico' (Juliano and Cuevas, 1932) and 'Olour' and 'Cambodiana' (Maheshwari *et al.*, 1955) produce only nucellar embryos. Phenotypic differentiation of nucellar seedlings from the zygotic seedlings is extremely difficult (Schnell *et al.*, 1994), while genotypic differentiation is possible through isozyme analysis (Degani *et al.*, 1990, 1992, 1993; Schnell and Knight, 1992). Nucellar seedlings are genetically identical to their parents and hence all progeny from such cultivars are theoretically alike; however, because mango has been in cultivation for a long period of time, somatic mutations possibly have occurred in some southeast Asian cultivars. The mutations probably are masked by the polyploid nature of the genome. Gan *et al.*, (1981) compared the isozymes of

grafted scions of polyembryonic mango of the same cultivar from different parts of Malaysia and found that there were differences in the banding patterns. 'Carabao,' 'Cecil,' 'Philippine' and 'Manila' show striking similarity and they are all believed to have originated from the Philippines. In Indonesia, there are several 'Aroemanis' selections that are differentiated numerically, e.g., 'Aroemanis 1,' 'Aroemanis 2,' etc., but the differences among these selections are subtle (Mukherjee, in press). These 'cultivar groups' provide strong evidence that somatic mutations have occurred at some point during cultivation from a common ancester.

Mango embryos do not have a distinct rest period and cannot tolerate partial dehydration. Embryo development and germination are continuous. Hence, they are considered to be recalcitrant and cannot remain viable for more than a few days (or 1-2 weeks) in storage at ambient temperatures (Parisot, 1988). Recalcitrance of mango seeds would have prevented natural dissemination of the species outside its native land.

2.2 Anthracnose

2.2.1 Importance and Distribution

Colletotrichum and its teleomorph *Glomerella* have been implicated in plant diseases, generally referred to as anthracnose, which is the major pre- and post-harvest problem of several tropical fruits including mango (Bailey and Jeger, 1992). The fungus, *Colletotrichum gloeosporioides* Penz., has been identified as the causal agent of mango anthracnose (Fawcett, 1907), although *C. acutatum* and *C. gloeosporioides* var. *minor* are also implicated in the disease in Australia (Cook, 1975).

The fungus has a worldwide distribution, but is most abundant in tropical and subtropical regions. It can persist in seed or other plant debris as well as thrive on weed hosts. *Colletotrichum gloeosporioides* also has the ability to exist as a saprophyte in necrotic tissues which serve as a constant source of inoculum (Simmonds, 1965). The fungus has rarely been isolated from the soil (Barker and Pitt, 1987).

Anthracnose of mango, also known as blossom blight, leaf spot or fruit rot, is the most widespread fungal disease of all mango growing countries (Prakash and Srivastava, 1987; Jeffries *et al.*, 1990; Nishijima, 1993; Ploetz and Prakash, in press). It was first reported in Puerto Rico (Collins, 1903) and very soon thereafter in Florida (Fawcett, 1907). While blossom blight is considered to be the most destructive form of anthracnose in drier mango growing countries like India (Prakash and Srivastava, 1987), postharvest infection is considered to be the most serious threat in the humid tropics (Jeffries *et al.*, 1990). The prevalence and severity of the disease are influenced by heavy rains, dew and high relative humidity (Quimo and Quimo, 1975; Prakash and Raoof, 1985; Hamilton, 1993).

2.2.2 Taxonomy

The taxonomic history of *C. gloeosporioides* is very confusing. The fungus was first described as *C. gloeosporioides* by Penzig in 1887 (Arx, 1970). It is a member of the form order Melanconiales in the form class Deuteromycetes. It is the anamorphic state of *Glomerella cingulata* (Stonem) Spauld. & V. Schr., an Ascomycete of the family Polystigmataceae (Arx, 1970; Alexopoulus and Mims, 1979). Species differentiation is mostly based on conidial measurements, since the perfect stage occurs only very rarely. This has led to the description of almost 750

species between the teleomorph and anamorph (Arx, 1970); however, it is highly probable that many are synonyms (Sutton, 1980). Sutton (1992) described 39 recognizable species, although he cautions that the list may not be comprehensive. The notoriously variable nature of both *Glomerella* and *Colletotrichum* (Hindroff, 1975) and their unstable nature in culture, even in single spore isolates (Wheeler, 1956; Sutton, 1992), could lead to more species being described. The application of molecular techniques and genome analysis in recent years is expected to reduce the existing confusion and enhance our understanding of the genetic complexity of the pathogen (Manners *et al.,* 1992; Rodriguez and Redman, 1992).

2.2.3 Description of the Fungus

In culture, the mycelium of *C. gloeosporioides* is fine, fairly dense and aerial, usually of low elevation. From above, the overall color is white to light grey, although occasionally the mycelium appears darker. From below, the substrate may be dark or light at the center with a pinkish margin (Mordue, 1971).

The conidia are apical, thin-walled, hyaline and aseptate; however, just prior to germination a septum develops. Setae may be present or absent. Usually the conidia appear pale salmon to pinkish *en masse*. Individual conidia are straight, cylinderical with an obtuse apex and truncate base (Sutton 1980; Baxter *et al.*, 1983; Holliday, 1989).

2.2.4 Infection Process

After attachment to the cuticle, the conidia germinate within 12-48 h. Usually they become uniseptate just prior to germination. The germ tube grows only for a very short distance (10-20 μm) (Jeffries *et al.,* 1990). Formation of an

appressorium is highly characteristic of the genus (Bailey *et al.,* 1992). The young appressorium or the protoappressorium is lightly pigmented or hyaline, but the walls become thicker and darker with age (Emmet and Parberry, 1975).

The mechanism by which *C. gloeosporioides* penetrates plant surfaces has been a subject for debate. Natural openings such as stomata, wounds and insect damage would facilitate entry of the fungus into the host tissue (van der Bruggen *et al.,* 1990); however, *C. gloeosporioides* appears to infect the tissues by direct penetration. Three possible mechanisms of direct penetration have been proposed. These are based on 1) mechanical force, 2) enzymatic digestion and 3) a combination of both (Bailey *et al.,* 1992). Studies on the formation of the infection peg and subsequent melanization favor the mechanical entry of the fungus into the host tissue (Mercer *et al.,* 1971; Suzuki *et al.,* 1982; Wolkow *et al.,* 1983; Katoh *et al.,* 1988; O'Connell *et al.,* 1992). Penetration of the fungus into the host tissue without appressorium formation suggests an enzymatic digestion of the host tissue before entry (Chau and Alvarez, 1983; Porto *et al.,* 1988). In addition, identification of cuticle and cell wall degrading enzymes such as cellulases, cutinases, pectinases, pectin lyase and polygalacturonases in the culture medium (Dickman *et al.,* 1982; Kolattukudy, 1985; Prusky *et al.,* 1989; Gantotti and Davis, 1993) and the inability of cutinase deficient mutants of the fungus to successfully penetrate intact host-tissues (Dickman and Patil, 1986) also favor the enzymatic penetration of the fungus. It is probable that the fungus deploys one or both mechanisms to gain entry into the host at specific situations which are not clearly understood at this time.

2.2.5 Phytotoxins

Despite the extensive damage done by *Colletotrichum* spp., only a very few studies have been undertaken to study the involvement of phytotoxic metabolites in the disease process. Three different classes of metabolites have been isolated from the culture filtrate produced *in vitro*. Aspergillomarasmins (= lycomarasmic acid) have been isolated from *C. gloeosporioides* causing anthracnose of olives (*Olea europea* L.) (Ballio *et al.*, 1969); colletopyrones from *C. nicotianae* (Gohbara *et al.*, 1976) and colletotrichins from *C. capsici* and *C. nicotianae* (Grove *et al.*, 1966; Gohbara *et al.*, 1978). In addition, culture filtrates of *C. gloeosporioides* from several hosts such as lime (*Citrus aurantifolia* L. Swingle), northern jointwitch, *Stylosanthes* and mango have been shown to exhibit phytotoxic activity (Sharma and Sharma, 1969; Walker and Templeton, 1971; Sahni *et al.*, 1974; Szabados *et al.*, 1987; Litz *et al.*, 1991). All the three isolated metabolites inflicted anthracnose-like symptoms on different plant species suggesting the nonspecific nature of these metabolites.

2.2.6 Symptom Development

Typical infection by the fungus involves a sequence of events, culminating in disease symptom (Dodd *et al.*, 1989). The inoculum is deposited on the fruit, inflorescence or leaf surface as a water splash-borne conidium, and adheres to the plant cuticle (Jeffries *et al.*, 1990). The pathogen attacks the young leaves more often than the old leaves. After the fungal infection is initiated, depressed dark brown to black spots are seen on the leaf surface (Prakash and Srivastava, 1987). The spots enlarge into lesions, which coalese and destroy large areas; however, in most cases severely affected leaves become curled or distorted (Jeffries *et al.*,

1990). Development of a distinct halo around the lesion has not been reported for *C. gloeosporioides*; however, halo formation has been observed in *C. lagenarium* (Suzuki *et al.*, 1983). In addition, Suzuki *et al.* (1983) have also observed that halo formation occurs around the penetration sites due to the action of cellulase produced by the fungus and cycloheximide prevents the formation of penetration hyphae and halo formation.

2.2.7 Quiescent Infection

The term 'quiescent infection' describes a dormant parasitic relationship which, after a time, changes to an active one (Swineburne, 1983). It appears that the host exhibits a state of 'temporary resistance' to the pathogen. Quiescent infections of anthracnose fungus in mango fruits is the most serious disease in mango. It is suggested that the unripe fruits retard or arrest the growth of the fungus by producing certain preformed fungitoxic compounds (e.g., dienes, epicatechins) (Prusky *et al.*, 1982, 1985, 1988; Adikaram, 1981) or phytoalexins (Brown and Swineburne, 1980). In addition, the fungus may not be able to utilize its full enzyme potential due to unavailability of nutrition from the immature fruits (Jeffries *et al.*, 1990). As the fruit ripens, these barriers are broken and the fungus invades the fruit tissue rapidly. In mango, field inoculation of unwounded fruits at several stages of maturity has not caused disease symptoms; however, once the fruits are detached from the trees, symptoms develop as early as three days (Daquioag and Quimo, 1979; Jeffries *et al.*, 1990). Typically the lesions develop in a streak extending from the fruit stalk downwards, usually referred to as the 'teardrop' symptoms.

The fruit can be attacked during any stage of its development. Anthracnose on young fruits often results in very low fruit set (Hamilton, 1993); however, latent

infections of the mature fruits in the humid tropics severely limit the export market (Derbyshire and Shipway, 1978). In addition, the economic loss due to quiescent infection is much greater than field losses, as harvesting, handling, packing and shipping costs are added (Jeger and Plumbley, 1988).

2.3 Breeding for anthracnose resistance in mango

Conventional breeding of mango has been practiced for only three decades, often with the idea of fruit quality improvement. Most of the existing cultivars are ancient seedling selections and there is considerable resistance to accept new cultivars, particularly in India. Controlled crosses have been attempted since the late seventies in India (Singh *et al.*, 1980), as a result of which a few hybrids, such as 'Mallika,' 'Amrapalli' and 'Ratna' were released. In most of these hybridization programs, the aim was to address either alternate bearing or fruit quality. The success of conventional breeding is severely limited by poor fruit set, heterozygosity of parents, long juvenile period of the seedling and prevalence of polyembryony (Lavi *et al.*, 1989; Mathews and Litz, 1992; Iyer and Degani, in press). There is no breeding program whose primary goal is to impart disease resistance, mainly due to the lack of knowledge of the genetics of disease resistance (Iyer and Degani, in press).

It is believed that the polyembryonic southeast Asian cultivars are generally more resistant to anthracnose than the monoembryonic Indian cultivars; however, it also appears that anthracnose resistance is dependent on environment (Nishijima, 1993). For instance, 'Carrie' is rated as a resistant cultivar in Australia (Peterson, 1984) and Florida (Knight, 1993) whereas it is susceptible in the Philippines (Pordesimo *et al.*, 1983). 'Carabao' is susceptible in Florida (Knight, 1993) and

resistant in Australia (Peterson, 1984). The polyembryonic cultivars originated in the rainforests of southeast Asia; hence, they probably developed anthracnose resistance as a survival mechanism, since the conditions there are optimum for anthracnose. Among the commercial mango cultivars, 'Tommy Atkins' is considered to be the most anthracnose resistant (Peterson, 1984; Knight, 1993; Iyer and Degani, in press). In addition, 'Parish' and 'Fairchild' have been reported to be relatively resistant (Yee, 1958); however, these two cultivars are not grown on a commercial scale. All these cultivars arose as open pollinated seedling selections mainly based on their fruit quality.

Interspecific crosses have rarely been attempted in mango. Sexual compatability exists between *M. indica* and *M. foetida,* and *M. zeylanica* (Mukherjee *et al.,* 1968). Anthracnose resistance appears to exist in *M. laurina,* a species closely related to *M. indica* (Bompard, 1993); however, because of the difficulties in achieving controlled pollination and the limited success attained so far, it would be impractical to attempt interspecific hybridization in mango for elevating anthracnose resistance.

In vitro approaches offer potential for achieving specific breeding objectives in cultivars of perennial species. Disease-resistant genotypes have been recovered following *in vitro* selection, e.g., peach (*Prunus persica* L.) (Hammerschlag, 1988) and lemon (*Citrus limon* (L.) Burm.) (Gentile *et al.,* 1992a). With respect to *in vitro* selection, Hammerschlag (1992) points out that it is possible to exercise greater control of the culture conditions and to screen a large number embryogenic cells in a short time, which is a great advantage in perennial species breeding. An efficient regeneration system combined with an effective selective agent makes *in vitro* selection for disease resistance a more viable approach than conventional

breeding (Daub, 1986; van den Bulk, 1991; Hammerschlag, 1992). The availability of an effective regeneration system (DeWald *et al.*, 1989a,b; Litz *et al.*, 1993) and selective agent (phytotoxin or the culture filtrate containing the phytotoxin) suggest that *in vitro* selection could be used for developing anthracnose resistance in mango cultivars.

The current study was carried out with the following objectives: 1) to select resistant embryogenic cultures of mango cv. 'Hindi' and 'Carabao' using the phytotoxic metabolite produced by *C. gloeosporioides*; 2) to characterize the nature of resistance in the selected lines at the protein and DNA level, using electrophoresis and random amplified polymorphic DNA (RAPD); 3) to achieve somatic embryogenesis from the selected embryogenic lines.

CHAPTER 3
PARTIAL PURIFICATION OF A PHYTOTOXIC METABOLITE FROM *COLLETOTRICHUM GLOEOSPORIOIDES* ISOLATED FROM MANGO

3.1 Introduction

Several bacteria and fungi have been shown to produce phytotoxins, which are implicated in the disease process (Rudolph, 1976; Yoder, 1980; van Alfen, 1989). Several plant pathogenic fungi, such as *Alternaria* spp. (Templeton, 1972), *Cercospora* spp. (Daub, 1986; Guchu and Cole, 1994), *Fusarium* spp. (Hiroe and Nishimura, 1956) and *Helminthorium* spp. (Yoder, 1980) have been shown to produce phytotoxins, which have been either partially or fully purified and characterized. Many species of the fungus *Colletotrichum* are phytopathogens and are the causal agent for the necrotic disease anthracnose (Jeffries *et al.*, 1990). *Colletotrichum gloeosporioides* Penz. is the causal agent of anthracnose of several tropical fruits, including mango (Waller, 1992).

Goodman (1960) isolated a phytotoxin, colletotin, from *C. fuscum* Laub. This toxin alters the permeability of the plant host cells, thereby causing loss of foliar integrity (Lewis and Goodman, 1962). Since then, several other species of *Colletotrichum* such as *C. atramentarium* (Wagner, 1961), *C. capsici* (H. Syd.) E. Butl. and Bisby (Narain and Das, 1970; Nair and Ramakrishnan 1973), *C. falcatum* Went, *C. gossypii* Southw. (Sahni *et al.*, 1974), *C. musae* (Berk. and M.A. Curtis) (Chen, 1968) and *C. nicotianae* (Gohbara *et al.*, 1978; Duke *et al.*, 1992) have been shown to produce phytotoxins that are involved in the disease process. *Colletotrichum gloeosporioides* has been demonstrated to produce phytotoxic metabolites either *in vitro* or *in vivo* in lime (*Citrus aurantifolia* L.) (Sharma and Sharma, 1969; Sahni *et al.*, 1974), northern jointwitch (*Aeschynomene virginica* L.)

Sharma, 1969; Sahni *et al.*, 1974), northern jointwitch (*Aeschynomene virginica* L.) (Walker and Templeton, 1972) olive (*Olea europaea* L.) (Ballio *et al.* 1969; Bosquet *et al.* 1971) and *Stylosanthes scabra* (Szabados *et al.*, 1987). Although a phytotoxin may also be implicated in mango anthracnose, no attempts have been made so far either to partially or fully purify a phytotoxin. In the present study, attempts were made to purify a phytotoxic metabolite produced by *C. gloeosporioides* isolated from mango.

3.2 Materials and Methods

Isolation of a virulent monoconidial culture (MCC) of *Colletotrichum gloeosporioides*. Young leaves showing anthracnose leaf spot symptoms were collected from trees of the cultivar 'Tommy Atkins' in the germplasm collection of Tropical Research and Education Center, Homestead. The leaves were incubated overnight in moist conditions within a polyethylene bag to facilitate sporulation of the fungus. Leaf pieces showing the disease symptoms were excised, washed in a 10% (v/v) Clorox solution and rinsed twice with sterile, deionized water. Small leaf pieces (1-2 cm^{-2}), with the cut passing through the leaf spot were placed on sterile potato dextrose agar medium (PDA) in 90x15 mm petri plates. The petri plates were sealed with Parafilm and incubated under a 16 h photoperiod at 27°C. Fungal colonies were clearly visible on the third day after inoculation. Mycelial discs from those colonies that resembled *C. gloeosporioides* were transferred to fresh PDA plates and allowed to sporulate under the same conditions.

The species was identified after microscopic examination of the spores. A small quantity of the spores from the fastest growing colonies was suspended in 1 ml of sterile water. Using a hemocytometer (Hausser Scientific, PA), the spore

concentration was determined. A dilution containing approximately 500 spores was plated on a water agar medium. Germinating spores were visualized after 16 h incubation under a stereo dissection microscope, removed individually using a sterile needle and were placed onto a fresh PDA medium. These single spore cultures developed into fresh colonies. Seventy-five such monoconidial cultures (MCC) were plated and from these, fifteen of the most rapidly growing colonies, assessed for their ability to grow quickly and to sporulate, were tested for virulence using a mango leaf bioassay.

Spores from the selected MCC were suspended in sterile deionised water and the concentration was adjusted to 5,000 spores ml^{-1}. Ten ml of this dilution was sprayed onto a fully expanded young leaf *in vivo* and the sprayed leaves were individually bagged and labelled. Sterile water sprays served as the control. The leaves were detatched from the trees 48 h after inoculation and kept under humid conditions in an incubator. After 48 h the spots that developed on the leaves were counted using a magnifying glass. The five most virulent strains were chosen and were retested in the same manner; 10 leaves were sprayed for each culture. From these results the most virulent MCC was identified and used in the subsequent studies.

Establishment of broth cultures. Spores from the isolated virulent strain (MCC #46) were suspended in sterile, deionized water and the density was adjusted to 10,000 spores ml^{-1}. The spores were germinated in Czapek-Dox liquid broth in 50 ml Erlenmeyer flasks. The broth was prepared by dissolving 35 g of the medium in 1.0 liter of deionized water and was dispensed in 20 ml aliquots in 50 ml Erlenmeyer flasks and autoclaved at 1.1 $kgcm^{-2}$ pressure at 120°C for 15 min. Each flask was inoculated with 100 µl of spore suspension and incubated on a rotary

shaker at 100 rpm under low light conditions. After 5 days the germinated spores in each flask were transferred along with the medium to a 250 ml Erlenmeyer flask; the final volume of the medium was adjusted to 100 ml with fresh, sterile Czapek-Dox liquid broth. After 1 week the contents from two flasks were transferred to a 1.0 liter fleaker containing 300 ml of fresh broth. The fungus was allowed to grow for 9 more days. After 21 days the cultures were filtered through a double layer of cheese cloth and the filtrate was refiltered through Whatman #1 filter paper. This final culture filtrate was used for the further purification of the phytotoxic metabolite.

Partial purification of phytotoxic metabolite from culture filtrate. Purification was carried out according to the protocol of Gohbara *et al.* (1978). Four liters of the culture filtrate was acidified to pH 3.0 using 1N HCl and extracted repeatedly with ethyl acetate (v/v) in a separating funnel. The aqueous layer was removed and the organic fraction was concentrated to 500 ml *in vacuo*. The concentrate was washed 1x with saturated sodium bicarbonate (v/v) and 2x with sterile deionized water to obtain the neutral extract. This neutral extract was dissolved in an equal volume of 80% (v/v) methanol and shaken 3x with n-hexane. The hexane layer was discarded. The aqueous methanol fraction containing the metabolites was measured and concentrated *in vacuo* to 20% of its original volume. This aqueous extract was filter-sterilized and used as the phytotoxic fraction in the experiments. Four liters of full strength Czapek-Dox broth was extracted in the same manner and used as an additional medium control.

Tests for phytotoxicity. Several bioassay systems are used to demonstrate the phytotoxic action of partially or fully purified compounds. The first test for phytotoxicity normally is to see if the purified fraction can induce either partial or

complete disease symptoms in the host. In addition, seeds or seedlings and tissue cultures are considered as an effective bioassay system (Rudolph, 1976).

Effect of phytotoxic metabolite on germination of lettuce seeds (Gohbara *et al.*, 1978). Seeds of lettuce 'Butter Crunch' (Page's Seed Company, Greene, NY) were used in this study. Seeds were disinfested in 20% (v/v) commercial bleach with a few drops of Tween 20 for 30 min and rinsed thoroughly several times with sterile deionised water. The seeds were then dried on a sterile paper towel in a laminar flow hood until the seeds were completely dry. Standard glass petri plates (90 x 15 mm) each containing a Whatman #1 filter paper were sterilized by autoclaving for 20 min at 120°C and 14 $kgcm^{-2}$ pressure. Five concentrations of phytotoxic metabolite, i.e., 0, 0.5, 1.0, 5.0 and 10.0% (v/v) were tested. An additional control with Czapek-Dox extract at 10.0% (v/v) was also used. A total volume of 10.0 ml liquid was poured in each plate. Twenty-five seeds were placed in each plate and four such plates were employed for each treatment. The petri plates were sealed with Parafilm and incubated at 26 ± 1°C with a 16 h photoperiod. Germination occurred after five days and continued until the 10th day. The number of seeds that germinated on the 10th day after incubation was used for analysis.

Effect of phytotoxic metabolite on germination of tobacco seeds (Templeton, 1972). Seeds of tobacco 'Havana' (J.L. Hudson Seeds, CA) were used in the study. Seeds were disinfested and dried as described above. Four concentrations of phytotoxic metabolite viz., 0, 1.0, 5.0 and 10.0% (v/v), were used in the study along with a medium control (Czapek-Dox extract) at 10.0% (v/v). Ten ml of the appropriately diluted phytotoxic metabolite was poured into each petri plate. Two hundred seeds were placed in each plate and the plates were incubated as described before. Five plates were employed for each treatment. Germination count

after 7 days of incubation was used for analysis. Root and shoot lengths were measured in 20 randomly selected seedlings that germinated in each plate.

Mango leaf bioassay. Fully expanded young leaves from 'Tommy Atkins' were used in this study. The effect of the phytotoxic metabolite was studied both *in vitro* and *in vivo*. Ten µl of the phytotoxic metabolite was pipetted onto the leaf lamina at the inter-veinal surface and a sharp prick was carefully made using a No. 2 pin. On the opposite side of the midrib in the same leaf, 10 µl of the medium control was pipetted and punctured as before. In the field the leaves were held flat until the liquid dried and were then bagged. Under *in vitro* conditions, the leaves were incubated after treatment under moist conditions. Five leaves were tested in each case, and two spots were made on each leaf. After three days the leaves were observed for symptom development in the regions of inoculation.

Effect of phytotoxic metabolite on mango proembryos. Embryogenic cultures were subjected to a dose response study with the phytotoxic metabolite. Actively growing embryogenic cultures from 'Hindi' and 'Carabao' were sieved through 960 mesh (Monsalud, 1994) and the fine fraction of the cultures that passed through the sieve were challenged with four concentrations of phytotoxic metabolite viz., 2.5, 5.0, 7.5 and 10.0% (v/v). An unchallenged control and an additional medium control with 10.0% (v/v) of Czapek-Dox extract were also included. The phytotoxic metabolite and the medium control were filter-sterilized and added to the autoclaved medium after cooling. Four replicates were maintained for each treatment. Approximately 100 mg of sieved cultures were inoculated into 10 ml medium in 50 ml Erlenmeyer flasks. Incubation was under low light conditions at 120 rpm and 25°C on a rotary shaker. After 1 week of challenge, putatively 'resistant' and 'susceptible' proembryonic masses were counted as follows.

Proembryonic masses that turned brown or black were counted as susceptible and those that were still creamy white were counted as resistant. Proembryonic masses contained in 100 µl of the medium were pipetted out and counted under the dissection microscope and five such counts were made from each flask and the mean was taken for analysis.

Statistical Analyses. Statistical analyses were carried out using the SAS (SAS Institute, Cary, NC). The data on seed germination were transformed using Fisher tables for Angular transformation values. General linear model was used to determine the regression coefficients.

3.3. Results

Effect of phytotoxic metabolite on germination of lettuce seeds. There was a significant reduction in the percentage of germination with increasing concentration of the phytotoxic metabolite. The high regression coefficient ($r^2 = 0.901$) indicates the effect of the phytotoxic metabolite on lettuce seed germination. Even at the lowest concentration of the phytotoxic metabolite, i.e., 0.5% (v/v), the germination percentage declined from 91% in the water control to 67%. There were no significant differences between 0.5% and 1.0% (v/v) of phytotoxic metabolite on germination of lettuce seeds. At 10.0% (v/v), germination was reduced to only 7% (Fig. 3.1a). Only the radicle emerged in the seeds exposed to 5.0 and 10.0% (v/v) of the phytotoxic metabolite. The cotyledons did not separate from the seed coat and eventually the germinated seeds died. There was no significant difference in germination of the seeds germinated in water and 10.0% (v/v) of medium control (purified Czapek-Dox extract), indicating the fungal origin of the phytotoxic metabolite.

Effect of phytotoxic metabolite on germination of tobacco seeds. A reduction in germination percentage was observed with increasing concentration of the phytotoxic metabolite. However, there was no significant reduction in germination between the seeds that germinated in water and in 1.0% (v/v) of phytotoxic metabolite. At 5.0% (v/v) phytotoxic metabolite, the reduction in germination was not as marked as in lettuce seed germination. The radicle emerged, but the cotyledons did not unfurl from the seed coat. Greening of the hypocotyl was seen in most of the germinated seeds, when they were germinated in 5.0% (v/v) phytotoxic metabolite. Germination was only 2.4% when the seeds were germinated in 10.0% (v/v) phytotoxic metabolite (Fig. 3.1b.) There was no appreciable reduction in germination of tobacco seeds when they were germinated in 10.0% (v/v) Czapek-Dox extract in comparison with water, giving further evidence of the fungal origin of phytotoxic metabolite (Table 3.1).

Both shoot and root growth were significantly inhibited in comparison with the control, when germinated in phytotoxic metabolite, irrespective of the concentration used (Table 3.1). Although seedlings that germinated in 1.0% (v/v) phytotoxic metabolite showed significant reduction in shoot and root growth, the seedlings were normal in appearance. Seeds that germinated in 5.0% (v/v) phytotoxic metabolite showed very poor root growth and shoots did not develop fully. At 10.0% (v/v) phytotoxic metabolite, the growth was completely arrested after radicle emergence (Fig. 3.2.). Seeds that germinated in 10.0% (v/v) medium control did not differ significantly from those that germinated in water.

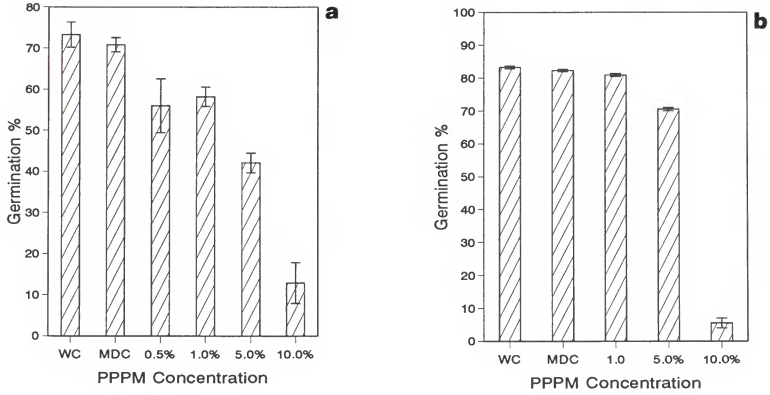

Fig. 3.1. Effect of partially purified phytotoxic metabolite (PPPM) on the germination of (a) lettuce seeds and (b) tobacco seeds.

Effect of phytotoxic metabolite on mango leaves. Chlorotic spots developed within 3 days of inoculation on leaf lamina, in the areas treated with the phytotoxic metabolite. These spots became necrotic in one week and later the center of the spots collapsed leaving a typical shot hole symptom resembling anthracnose of mango leaves (Fig. 3.3.). A transient chlorotic halo was observed around the spots only in the early stages of inoculation. There was no symptom development, except for the pin mark on the spots inoculated with Czapek-Dox extract.

Effect of phytotoxic metabolite on survival of proembryos. Susceptibility of proembryos increased with increasing concentrations of phytotoxic metabolite. The phytotoxic metabolite was toxic even at low concentrations (e.g., 1% v/v) and caused significant mortality of proembryos. Although the toxic fraction caused complete mortality at 10% (v/v), it is probable that the lethal concentration lies between 5 and 10% (v/v), since only about 30% of the proembryonic masses in both cultivars were tolerant to 5% phytotoxic metabolite. The medium control at 10% (v/v) caused only a marginal reduction in survival of proembryos (Table 3.2) which was not statistically significant. Both cultivars showed approximately equal sensitivity to the phytotoxic metabolite as indicated by overall cultivar means. The very high regression coefficients of 0.993 and 0.995 with 'Hindi' and 'Carabao,' respectively, also indicate the sensitivity of proembryos to increasing concentrations of the partially purified phytotoxic metabolite.

Table 3.1. Effect of partially purified phytotoxic metabolite (PPPM) on tobacco seed germination. Inhibition of root and shoot growth.[1]

TREATMENT	Root Length (mm)[2]	Shoot Length (mm)[2]
Water Control	6.45 ± 0.17a	3.03 ± 0.06a
Medium Control	6.20 ± 0.16a	2.93 ± 0.04ab
PPPM 1.0%	5.20 ± 0.16b	2.80 ± 0.07b
PPPM 5.0%	1.65 ± 0.06c	0.63 ± 0.05c
PPPM 10.0%	0.00d	0.00d
Regression Coefficient (r^2)	0.956	0.974

[1] Values shown are mean ± standard error
[2] Means are separated by Duncan's Multiple Range test. Means with the same letter do not differ significantly at p=0.05.

ANOVA

Source	DF	Sum of Squares	Mean Square	'F' value	Pr > F
Root length					
Treatment	4	675.100	168.775	518.89	0.0001
Error	95	30.900	0.325		
Corrected Total	99	706.000			
Shoot length					
Treatment	4	167.175	41.794	879.87	0.0001
Error	95	4.513	0.048		
Corrected Total	99	171.688			

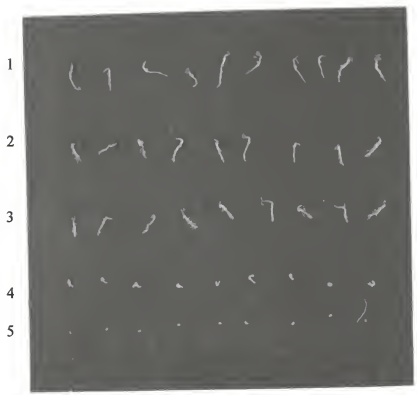

Fig. 3.2. Effect of partially purified phytotoxic metabolite on the germination of tobacco seeds. [Legend. 1. Water Control, 2. Medium Control, 3. PPPM 1% (v/v), 4. PPPM 5% (v/v), 5. PPPM 10% (v/v)]

Fig 3.3. Effect of partially purified phytotoxic metabolite on mango leaf.

Table 3.2. Percentage of resistant proembryos 1 week after cocultivation with partially purified phytotoxic metabolite(PPPM) from *Colletotrichum gloeosporioides*.[1]

Treatment [PPPM %(V/V)]	HINDI[2]	CARABAO[2]
Control	83.43 ± 1.76a	82.59 ± 0.56a
Czapek-Dox Extract 10%	82.90 ± 1.86a	81.41 ± 0.73a
PPPM 1.0%	76.68 ± 0.91b	71.73 ± 0.94b
PPPM 2.5%	65.39 ± 0.83c	61.47 ± 0.83c
PPPM 5.0%	36.87 ± 1.04d	34.52 ± 1.05d
PPPM 10.0%	0.0[e]	0.0[e]
Regression Coefficient (r^2)	0.993	0.995

[1]Values shown are mean transformed values ± standard error.
[2]Means are separated by Duncan's multiple range test Means with same letter do not differ significantly at p=0.05.

ANOVA

Source	DF	Sum of Squares	Mean Square	'F' value	Pr > F
'Hindi'					
Treatment	5	27395.093	5479.019	719.43	0.0001
Error	24	182.778	7.616		
Corrected Total	29	27577.870			
'Carabao'					
Treatment	5	26121.239	5224.248	1791.84	0.0001
Error	24	69.974	2.916		
Corrected Total	29	26191.213			

3.4 Discussion

In vitro production of phytotoxins in culture medium has been reported for several fungi (Rudolph, 1976; van Alfen, 1989), including several species of *Colletotrichum*. While some of these phytotoxins, such as helminthosporin (Gengenbach *et al.*, 1977) are host-specific, all of the *Colletotrichum* toxins have been reported to be nonspecific (Sahni *et al.*, 1974; Gohbara *et al.*, 1978; Duke *et al.*, 1992). The present study also corroborates this view, since the partially purified phytotoxic metabolite was also toxic to two other nonhosts, lettuce and tobacco.

Confirmation that a phytotoxin is involved in a disease is important, since many phytotoxins have been shown to be useful agents for *in vitro* selection for disease resistance (Daub, 1986; van den Bulk, 1991; Hammerschlag, 1992). Although crude culture filtrates can also be potential selective agents as demonstrated by Hammerschlag (1984; 1988) using *Xanthomonas campestris* pv. *pruni* against peach (*Prunus persica* L.), the involvement of secondary metabolites can confuse the results. Such false positives have been reported in *Citrus*, where an inverse correlation was observed between field resistance and culture filtrate resistance, when protoplasts were selected against *Phytophthora citrophthora* culture filtrate (Vardi *et al.*, 1986).

Nonspecific toxins such as those produced by *Colletotrichum* spp. are considered to be of great value for *in vitro* selection procedures, as they often lead to the recovery of plants with novel resistance factors (van den Bulk, 1991). Most of these non-specific toxins are generally regarded as virulence factors and plantlets regenerated folowing *in vitro* selection against these toxins show enhanced tolerance rather than absolute resistance (Pauly *et al.*, 1987). Success in the utilization of nonspecific toxins for *in vitro* selection depends on establishing the

level at which *in vitro* selection should be carried out. For instance Nachmias *et al.* (1990) reported the ineffectiveness of *Verticillium dahliae* (VD) toxin on the protoplasts of susceptible genotypes of potato. Wolf and Earle (1990) could not recover maize callus resistant to *Helminthosporium carbonum* race 1 toxin. Hence it has been suggested that an understanding of the mode of action of the toxin helps to improve the efficiency of the *in vitro* selection protocol (Daub, 1986; van den Bulk, 1991). Toxins from *Colletotrichum spp.* such as colletotin (Lewis and Goodman, 1962), aspergillomarasmin A (Ballio *et al.* 1969) and colletotrichins (Duke *et al.* 1992) have been shown to be active at the cellular level and to cause rapid membrane damage to plant cells.

The present study indicates that the fine fraction of rapidly proliferating proembryonal masses can be used for *in vitro* selection of mango against phytotoxic metabolites. The development of necrotic spots following inoculation of mango leaves is similar to the effect described by Gohbara *et al.* (1978) and Duke *et al.* (1992) by colletotrichins on tobacco leaves, indicating a similar mode of action at the cellular level in mango. The ineffectiveness of the purified Czapek-Dox extract in all the bioassays tested, confirms the fungal origin of the phytotoxic metabolite reported by Walker and Templeton (1978) with a wide range of hosts.

Fungal culture filtrate contains several compounds other than the phytotoxin. For instance, Vardi *et al.* (1986) identified auxin like substances from the culture filtrate of *Phytophthora citrophthora*. Hence, it is necessary to purify the culture filtrate at least to a certain extent and demonstrate that the purified fraction retains phytotoxic activity. Several assay systems are used as criteria to evaluate phytotoxins as factors in pathogenesis (Rudolph, 1976; Yoder, 1980). In addition, these assay systems are also helpful in determining whether the toxin is host-specific

or nonspecific. The first test for phytotoxicity is the induction of partial or complete disease symptoms upon infiltration in the intact host (Wheeler, 1975; Rudolph, 1976). Inhibition of seed germination and seedling growth are also considered as useful bioassays to demonstrate phytotoxicity (Vidyasekaran, 1972; Gohbara et al., 1978; Yoder, 1980). More recently plant tissue cultures are used to test the phytotoxicity of the purified fraction (van Alfen, 1989; Wedge *et al.,* 1995). In the current study, partially purified phytotoxic metabolite caused disease symptoms in mango leaves, inhibited the germination of lettuce and tobacco seeds and also exhibited a typical dose-response with embryogenic cultures of mango. These evidences suggest that the purified culture filtrate contains the phytotoxic metabolite produced by *C. gloeosporioides.*

Phytotoxins can play a key role in disease resistance breeding through somaclonal variation (Carlson, 1973; Reisch, 1983; Scowcroft *et al.*, 1983; Hammerschlag, 1992). The current study demonstrates that embryogenic cultures of mango consisting of proliferating proembryonic masses can be challenged successfully with the phytotoxic metabolite of *C. gloeosporioides.*

CHAPTER 4

EMBRYOGENIC MANGO CULTURES SELECTED FOR RESISTANCE TO CULTURE FILTRATE OF *COLLETOTRICHUM GLOEOSPORIOIDES* SHOW ENHANCED RELEASE OF EXTRACELLULAR ANTIFUNGAL PROTEINS. I. *In vitro* Selection and Dual Culture.

4.1 Introduction

Anthracnose is the major disease and production problem of mango (*Mangifera indica* L.), and is caused by the fungus *Colletotrichum gloeosporioides* Penz. Most commercial mango cultivars are highly susceptible to this disease. While it is widely believed that the polyembryonic Southeast Asian cultivars are more resistant to this disease than the monoembryonic Indian cultivars (Mathews and Litz, 1992), many of these cultivars appear to be susceptible in different regions (Knight, 1993), indicating the absence of true genetic resistance to this disease. The use of conventional breeding to introduce greater resistance to anthracnose in improved mango cultivars has been futile due to extremely poor fruit set, long juvenile period, unpredictable behaviour of F_1 progeny due to the heterozygosity among cultivars and polyembryony. *In vitro* selection for enhancing resistance to anthracnose at the cellular level could be a useful strategy for inducing disease resistance in existing mango cultivars

Using *in vitro* selection, it is possible to screen large populations of physiologically uniform cells under defined conditions (Hammerschlag, 1992). This enables selection pressure to be applied (using an appropriate selective agent) to a large number of cells having morphogenic potential. One of the most common traits that has been identified in somaclonal variants is disease resistance, identified by means of selection for resistance *in vitro* or simply by evaluating large numbers of unselected regenerants in the field for resistance. Although recovery of disease

unselected regenerants in the field for resistance. Although recovery of disease resistant plants by *in vitro* selection using appropriate selective agents has been reported for several herbaceous species (Daub, 1986; Galun and Breiman, 1992), this approach has rarely been applied to perennial or woody species. Successful recovery of disease resistance following *in vitro* selection has been achieved in only a few perennial species (Viseur 1986; Nadel and Spiegal-Roy 1987; Hammerschlag and Ognjnov 1990; Raman and Dhillon, 1990 and Rosati *et al.,* 1990). The most comprehensively documented studies have involved peach (*Prunus persica* L.) and lemon (*Citrus limon* L.). Hammerschlag (1988; 1990) screened embryogenic cultures derived from immature zygotic embryos of peach for resistance against the bacterial pathogen *Xanthomonas campestris* pv. *pruni.* Gentile *et al.* (1992a, b) regenerated *mal secco* resistant lemon, by selecting nucellar cultures against a partially purified phytotoxin produced by the causal fungus *Phoma tracheiphila.*

Other studies have been conducted to screen and select for resistance to various stress factors such as drought tolerance (Ochatt and Power, 1989) and salt tolerance (Kochba *et al.*, 1982; Spiegel-Roy and Ben-Hayyim, 1985; Ben-Hayyim and Goffer, 1989 and Sala *et al.,* 1990). Herbicide tolerance has been reported in tobacco (Chaleff and Keil, 1981) and *Citrus* (Spiegel-Roy *et al.,* 1983) using *in vitro* selection.

Somaclonal variants of *Musa spp.* showing improved resistance to *Fusarium oxysporium* f. sp. *cubense* race 4 (Hwang, 1985, 1990; Hwang and Ko, 1987) and strawberry showing resistance to *Fusarium oxysporium* f. sp. *fragerí* (Toyoda *et al.,* 1991) were recovered following field evaluation of plants regenerated from *in vitro* culture. Somaclonal variants of poplar resistant to *Septoria musiva* (Ostry and

Skilling, 1988) and leaf rust caused by *Melampsora medusae* (Prakash and Thielges, 1989) have also been reported.

One of the most common agents that has been used for screening and selecting perennial crops for disease resistance is the crude culture filtrate produced by a pathogen (Hammerschlag, 1984 and Viseur and Tapia y Figueroa, 1987). Hammerschlag (1992), however, has emphasized that, when possible, the purified phytotoxin should be used as the selective agent, as the possible interference of secondary metabolites produced by the pathogen could thereby be avoided. The interference of secondary metabolites was demonstrated in two studies with *Citrus* (Vardi *et al.*, 1986; Gentile *et al.*, 1990), where sensitivity to the culture filtrate was negatively correlated to the *in vivo* response of the cultivars used in the study.

Colletotrichum gloeosporioides causing anthracnose in olive (*Olea europaea* L.) (Ballio *et al.*, 1969), lime (*Citrus aurantifolia* L.) (Sharma and Sharma, 1969) and willows (Bosquet *et al.*, 1971) has been demonstrated to produce phytotoxic compounds *in vitro* and *in vivo*. These compounds were shown to be non-specific in their action against plant tissue (Sahni *et al.*, 1974; Walker and Templeton, 1978). Phytotoxins have also been purified and characterized from *C. fuscum* (Goodman, 1960) and *C. nicotianae* (Gohbara *et al.*, 1978) and are non-specific and active at the cellular level in the disease process (Lewis and Goodman, 1964; Duke *et al.*, 1992)

In order to achieve effective *in vitro* selection for disease resistance, an efficient regeneration system is required. Although studies to establish tissue cultures of mango were attempted as early as 1958 (Maheswari and Rangaswamy, 1958), currently, the established protocols are based upon those of Litz *et al.* (1982, 1984) who successfully demonstrated somatic embryogenesis from the nucellus of

polyembryonic mango cultivars and later monoembryonic mango cultivars (Litz, 1984) and of Dewald *et al.* (1989a, b) who described the establishment of suspension cultures of mango. In this study, suspension cultures of 2 mango cultivars, 'Carabao' and 'Hindi,' have been recurrently selected with the culture filtrate and partially purified phytotoxic metabolite of *C. gloeosporioides*, in order to determine their efficacy for *in vitro* selection of resistant off-types.

4.2 Materials and Methods

Induction and maintenance of embryogenic cultures. Embryogenic cultures were initiated from the nucellus of immature seeds from young fruits of the polyembryonic mango cultivars 'Hindi' and 'Carabao.' Young fruits approximately 45 days post-anthesis were harvested from the germplasm collection of the USDA, Miami ('Carabao') and TREC, Homestead ('Hindi'). Following disinfestation for 30 min in 20% commercial bleach (Clorox) containing a few drops of Tween-20, fruits were thoroughly washed with sterile deionized water (Litz *et al.*, 1982, 1984; Litz, 1984). The fruits were bisected under aseptic conditions using a #22 scalpel and the ovules were removed. The ovules were bisected longitudinally using a surgical knife (#10) and split into two equal halves on a sterile paper towel. The embryo masses were carefully removed and discarded. Since the excission of the nucellus from the integuments can damage the nucellus, the ovular halves were placed on the plant growth medium, so that the nucellus was in direct contact with the medium. The plant growth medium consisted of B5 major salts (Gamborg *et al.*, 1969), MS minor salts (Murashige and Skoog, 1962) supplemented with 400 mgl^{-1} of glutamine, 60 gl^{-1} of sucrose as a carbon source and 1 mgl^{-1} of 2,4-D. Gel-Gro (ICN Biochemicals,) @ 1.8 gl^{-1} was added as the gelling agent, after adjusting the pH to

5.8. The medium was autoclaved at 1.1 kg cm^{-2} and 120°C for 20 min. After sterilization, the medium was cooled to about 40-45°C and dispensed in 90x15 mm petri plates. The cultures were incubated in darkness at room temperature (27±2°C). To avoid accumulation of phenolic compounds in the plant growth medium, the explants were subcultured after three days. Thereafter, they were subcultured at three-week intervals. Proembryonic masses were established from the nucellar cultures in approximately eight weeks with 'Hindi' and ten weeks with 'Carabao'. After proliferation for 12 weeks on semisolid medium, suspension cultures were initiated by transferring approximately 2.0 g of proembryonic masses into 40 ml of plant growth medium of the same composition (without Gel-Gro) in 125 ml Erlenmeyer flasks. Suspension cultures were maintained on a rotary shaker in semi-darkness at 120 rpm at 25°C. Embryogenic suspension cultures were transferred weekly to fresh medium of the same composition in order to avoid the accumulation of phenolic compounds in the medium. Cultures from both of these cultivars were used in all reported studies, unless indicated otherwise.

Isolation of a virulent monoconidial culture (MCC) of Colletotrichum gloeosporioides and partial purification of phytotoxic metabolite from culture filtrate. [Refer also to Chapter 3, Materials and Methods, page 17.]

Determination of lethal concentration of culture filtrate. Embryogenic cultures growing in suspension were sieved through nylon screen with 1,000 µm mesh (Monsalud, 1994) and the fine fraction was used in the recurrent selection studies. Five concentrations viz., 10, 20, 40, 60, 80% (v/v), of culture filtrate were studied for their effect on the cultures. The culture filtrate was filter-sterilized and diluted with autoclaved medium under aseptic conditions. The total volume of the medium was 20 ml in 50 ml Erlenmeyer flasks. A set of unchallenged proembryonic

masses served as the control. Approximately 200 mg of the fine fraction of an embryogenic culture that passed through the sieve were challenged in each flask. Each treatment was replicated five times and the entire experiment was repeated twice. The cultures were incubated on a rotary shaker at 120 rpm under low light conditions for 1 week at 25°C. After one week, 100 µl of this suspension was pipetted and examined under a dissection microscope to assess the proportion of putatively tolerant and susceptible proembryos. Since all the proembryos were creamy white at the start of cocultivation, proembryos that became necrotic were counted as susceptible and the ones that were partly or fully creamy white were counted as putatively resistant. Five such counts were made from each flask and the mean was taken for analysis.

Recurrent selection of culture filtrate-resistant lines with progressively increasing concentrations of culture filtrate. Embryogenic cultures were sieved and challenged with culture filtrate as described above. Approximately 500 mg of the fine fraction of embryogenic cultures was challenged in each flask in a total volume of 40 ml medium. Concentrations tested were 0 (control), 20, 40, 60 and 75% (v/v) of culture filtrate. Seven flasks were maintained for each treatment and the control. After one week of challenge, the cultures were allowed to recover for 3 weeks in suspension culture medium without culture filtrate. At the end of the recovery phase, the cultures were subjected to the next higher concentration and the process continued. The lines that received the highest concentration of the challenge were proliferated in regular suspension culture medium. Putatively resistant and susceptible proembryos were counted as described above after the first and second recurrent selections only, as it was not possible to obtain a good count after the third challenge due to excessive browning of the entire cultures.

Development of culture filtrate resistant lines by continuous challenge. In this study, only 40% (v/v) of culture filtrate was used. Preparation of medium, sieving and cocultivation were done as described earlier in this section. Embryogenic suspensions were cocultivated with the culture filtrate for 4 weeks with weekly transfers to fresh medium of the same composition and 40% (v/v) culture filtrate. An additional control with 40% (v/v) of Czapek-Dox medium was included in the study. The broth was freshly prepared, filter-sterilized and the pH was adjusted to 5.8 and added to the medium after autoclaving and cooling. Seven flasks were employed for each treatment. After 4 weeks of cocultivation, embryogenic cultures were proliferated in regular suspension culture medium. Embryogenic cultures were weighed at biweekly intervals from week 0 to week 12.

Effect of phytotoxic metabolite on mango proembryos. [Refer to Chapter 3, Materials and Methods, page 21]

Development of phytotoxic metabolite resistant lines. Sieved embryogenic cultures were challenged with 7.5% (v/v) of phytotoxic fraction for three or four weeks with a weekly transfer to fresh medium of the same composition. There were two controls: an unchallenged control and an additional medium control with 10% (v/v) Czapek-Dox extract. Seven flasks were maintained for each treatment and the total medium volume for the flask was 15 ml. Cultures were incubated on a rotary shaker at 120 rpm for seven days. After three weeks of challenge, the cultures were proliferated in regular suspension culture. The resistant line thus obtained was designated as PTR (Phytotoxin Resistant Line). Embryogenic cultures were weighed biweekly during subculture until the 12th week.

Dual culture experiment. Dual culture experiments were performed as described previously by Storti *et al.* (1988) and Gentile *et al.* (1993) with some

modifications. Embryogenic cultures from actively proliferating suspensions of putatively resistant lines and unchallenged controls were filtered separately through sterile tissue paper. One gram of proembryonic mass were placed at opposite ends of a 90x15 mm petri plate, containing mango proliferation medium (DeWald *et al.*, 1989a). The petri plates were sealed with Parafilm and the cultures were incubated in darkness at room temperature (27±2°C) for four weeks. After four weeks, the culture from one side of the plate was removed and mycelial inoculum (≤5mm) was placed at the center of the plate. The plates were re-sealed with Parafilm and incubated in an incubator with 16 h photoperiod at 27°C. There were five plates for each line and the control. Growth of the fungus was monitored daily from the third day of dual culture until the 10th day. The diameter of the mycelial colony was measured at opposite sides and the mean diameter was taken into account for calculating the area of the colony using the formula $A = \pi r^2$. The experiment was repeated twice with both cultivars.

Conditioned medium experiment. Semi-solid mango proliferation medium was conditioned with 20% (w/v) of macerated embryogenic culture from the various putatively resistant lines and the unchallenged control. The medium was prepared as described earlier in this section. After autoclaving, the medium was allowed to cool to 50°C. Embryogenic cultures were rapidly cooled in liquid nitrogen and macerated under aseptic conditions. Macerated cultures were added to the cooled medium and the modified plant growth medium was plated in 90x15 mm petri plates. After 24 h, a mycelial inoculum was placed at the center of each plate and the plates were sealed with Parafilm. The plates were incubated under 16 h photoperiod in an incubator. Mycelial growth was determined from the diameter of the colony at opposite sides and the area was calculated. Observations were taken from the third

day of inoculation to the tenth day. Five plates were used for each treatment and the experiment was repeated twice.

Statistical analysis. All the data were subjected to statistical analysis using the SAS program (SAS Institute, 1982). The general linear model was used to compute the regression coefficients and the means were separated by Duncan's multiple range test.

4.3 Results

Selection of putatively resistant lines by progressively increasing culture filtrate concentrations. There was increased mortality of embryogenic cultures as the concentration of culture filtrate increased. Significant differences in the mortality of proembryos were observed between each concentration of culture filtrate tried during the first challenge in both cultivars. Complete mortality was achieved when challenged with 80% (v/v) culture filtrate. However, only 7.3% and 4.4% of the proembryos survived after exposure to 60% (v/v) culture filtrate in 'Hindi' and 'Carabao,' respectively. Hence, the lethal concentration lies between 60 and 80% (v/v) culture filtrate. Both cultivars were equally susceptible to various concentrations of culture filtrate. The regression coefficients were very high indicating the effect of increasing concentrations of culture filtrate on the resistance of proembryos (Table 4.1).

Challenged cultures were maintained in regular proliferation medium for three weeks before the second challenge. During the second challenge, the cultures

in various treatments were subjected to the next highest concentration of culture filtrate and cocultivated for 1 week. A general increase in resistance was observed with both cultivars (Table 4.2). For example, during the first challenge with 20% (v/v) culture filtrate, only 51.0% and 46.3% of the proembryos survived in 'Hindi' and 'Carabao.' respectively, while after the second challenge, 81.5% ('Hindi') and 52.0% ('Carabao') of the proembryos survived. Since 80% (v/v) culture filtrate was lethal, a sub-lethal concentration of 75% (v/v) was used to challenge the cultures that were selected with 60% (v/v) culture filtrate during the first challenge. Very few proembryos survived exposure to 75% (v/v) culture filtrate in both cultivars. Overall, 'Hindi' showed greater tolerance than 'Carabao' during the second challenge. There were fewer proembryos at 75% (v/v) culture filtrate that appeared to be resistant in 'Hindi' in comparison with 'Carabao'; however, it is possible that resistant proembryos could have been counted as susceptible. The proembryos were very dark, and all appeared necrotic after the second challenge. Hence, visual counting could not be done after the second challenge. The cultures from the line selected with 60% (v/v) culture filtrate initially followed by 75% (v/v) culture filtrate in the second challenge were recovered and designated as putatively resistant line I (PRL I).

Cultures from the other lines were proliferated for three weeks in regular suspension culture and were subjected to a third cycle of selection with the next highest concentration of culture filtrate. After one week of selection, cultures from

Table 4.1. Percentage of resistant proembryos 1 week after cocultivation with *Colletotrichum gloeosporioides* culture filtrate. First challenge.[1]

Treatment [CF % (v/v)]	HINDI[2]	CARABAO[2]
Control	81.68 ± 2.27a	77.18 ± 1.54a
10	55.08 ± 1.75b	53.28 ± 1.53b
20	43.85 ± 2.11c	42.86 ± 1.19c
40	30.22 ± 1.17d	33.09 ± 0.77d
60	15.59 ± 1.07e	11.65 ± 1.65e
80	0.0	0.0
Regression coefficient (r^2)	0.938	0.979

[1]Means expressed are transformed values ± standard error]
[2]Means with same letter do not differ by Duncan's multiple range test at p=0.05

ANOVA

Source	DF	Sum of Squares	Mean Square	'F' value	Pr > F
'Hindi'					
Treatment	5	21202.577	4240.515	334.80	0.0001
Error	24	303.984	12.666		
Corrected Total	29	21506.561			
'Carabao'					
Treatment	5	19693.209	3938.642	501.58	0.0001
Error	24	188.460	7.852		
Corrected Total	29	19881.669			

Table 4.2. Percentage of resistant proembryos 1 week after cocultivation with *Colletotrichum gloeosporioides* culture filtrate. Second Challenge.[1]

Treatment [CF % (v/v)]	HINDI[2]	CARABAO[2]
Control	76.21 ± 1.05a	81.58 ± 0.89a
10 → 20	64.68 ± 1.47b	46.15 ± 1.60b
20 → 40	62.02 ± 0.85b	36.85 ± 1.06c
40 → 60	52.66 ± 1.18c	26.46 ± 1.16d
60 → 75	1.45 ± 1.45d	8.44 ± 2.27e
Regression coefficient (r^2)	0.991	0.985

[1]Means expressed are transformed values ± standard error
[2]Means with same letter do not differ by Duncan's multiple range test at p=0.05

ANOVA

Source	DF	Sum of Squares	Mean Square	'F' Value	Pr > F
'Hindi'					
Treatment	4	17004.387	4251.097	568.06	0.0001
Error	20	149.672	7.484		
Corrected Total	24	17154.059			
'Carabao'					
Treatment	4	14777.619	3694.405	336.76	0.0001
Error	20	219.409	10.970		
Corrected Total	14	14997.028			

the line that received successive challenges with 40, 60 and 75% (v/v) culture filtrate, respectively, were recovered and designated as PRL II. After the fourth cycle of selection, line PRL III was recovered. However, none of the cultures survived the whole series of challenges from 10% to 75% (v/v) culture filtrate, and hence line PRL IV could not be established. The whole scheme of selection with progressively increasing concentration of culture filtrate is summarized in Fig. 4.1.

Development of culture filtrate resistant lines by continuous challenge. Significant reductions in the growth rates of embryogenic cultures treated with culture filtrate was observed with both cultivars in comparison with the controls. Proembryos in suspension culture show an initial lag phase for two or three weeks followed by exponential phase until the 10th week. Thereafter, the growth rate subsided. Unchallenged controls of both cultivars were subcultured at the 12th week. Prolonged maintenance in a single flask resulted in increased mortality of cultures, and hence they were subdivided during the next subculture. Embryogenic cultures recurrently selected with culture filtrate for 4 weeks showed an extended lag phase. 'Hindi' and 'Carabao' showed lag phases of five weeks and seven weeks, respectively. While the unchallenged controls entered log phase of growth after 12 weeks the selected cultures were still growing exponentially for 12 weeks and more. Significant reduction in growth was observed between the challenged cultures and the controls until the 10th week with 'Hindi' (Fig. 4.2 a) and throughout the study period in 'Carabao' (Fig. 4.2 b), thus indicating that 'Carabao' was much slower in recovering after challenge inoculation. Cultures that were putatively resistant after the recurrent selection were recovered and designated as PTL V. This study also reveals that the culture filtrate contains phytotoxic metabolite produced by the

fungus, since these cultures showed significantly slower growth than the cultures with 40% (v/v) Czapek-Dox broth (medium control) and the unchallenged controls.

Effect of phytotoxic metabolite on survival of proembryos. Mortality of proembryos increased with increasing concentrations of putative toxic metabolite. The phytotoxic metabolite was toxic at low concentrations (e.g., 1% v/v) and caused significant mortality of proembryos. Although the phytotoxic metabolite caused complete mortality at 10% (v/v) (see Table 3.2.), it is probable that the lethal concentration lies between 5 and 10% (v/v), since more than 30% of the cultures in both cultivars were tolerant to 5% toxic metabolite. Cultures selected with phytotoxic metabolite became dark more quickly in comparison with cultures selected with culture filtrate. The Czapek-Dox extract at 10% (v/v) caused only a marginal reduction in survival of proembryos, which was not significant. Both cultivars showed approximately equal sensitivity to the phytotoxic metabolite as indicated by the overall cultivar means. The very high regression coefficients of 0.993 and 0.995 with 'Hindi' and 'Carabao' respectively, also indicate the sensitivity of proembryos to increasing concentrations of the phytotoxic metabolite.

Development of phytotoxic metabolite resistant lines. Because 10% (v/v) of the phytotoxic metabolite was lethal and 5% (v/v) allowed more than 30% of the selected cultures to survive, the selection pressure was adjusted to 7.5% (v/v) of the phytotoxic metabolite. The growth of selected cultures of both varieties was minimal, when the cultures were exposed continously to selection pressure. Unchallenged and cultures challenged with 7.5% (v/v) of Czapek-Dox

Challenge No.	Culture Filtrate Concentration (% v/v)			
1	60	40	20	10
2	75	60	40	20
3		75	60	40
4			75	60
5				75
Recovered line	PRL I	PRL II	PRL III	PRL IV

Fig. 4.1. Selection of mango PEM with resistance to *C. gloeosporioides* culture filtrate

extract showed very similar growth patterns with both cultivars (Figs. 4.3 a & b). The initial lag phase of growth was two or three weeks, as observed earlier, followed by an exponential phase of growth of up to 10 weeks. Thereafter, the growth rate diminished as the cultures exceeded the capacity of the culture medium to support rapid growth. There was no difference between the two controls of both cultivars. On the other hand, challenged cultures of both cultivars were much more slow growing than the controls. Proliferation was evident two weeks after cessation of selection pressure, in the form of newly formed white secondary proembryos. None of the cultures of either cultivar survived four cycles of recurrent selection with the phytotoxic metabolite.

Dual culture. All of the selected lines except PRL I inhibited mycelial growth in dual culture. However, the lines PRL III, PRL V and PTR in both cultivars demonstrated significantly greater fungal inhibition than the other lines. The results show increased fungal inhibition in relation to an increase in exposure to selection pressure. Removal of the embryogenic culture that was established on one side of the petri plate before dual culture did not cause any alteration in fungal growth (Fig. 4.4). In control and less tolerant embryogenic lines the mycelial colony overgrew the embryogenic cultures and covered the entire plate. Overall, the cultivars showed no significant differences. The high regression coefficients in both cultivars (Table 4.3) also indicate increased fungal inhibition with respect to increase in the number of recurrent selections. The results also indicate the probable involvement of antifungal compounds that were produced by the selected lines in mycelial growth inhibition.

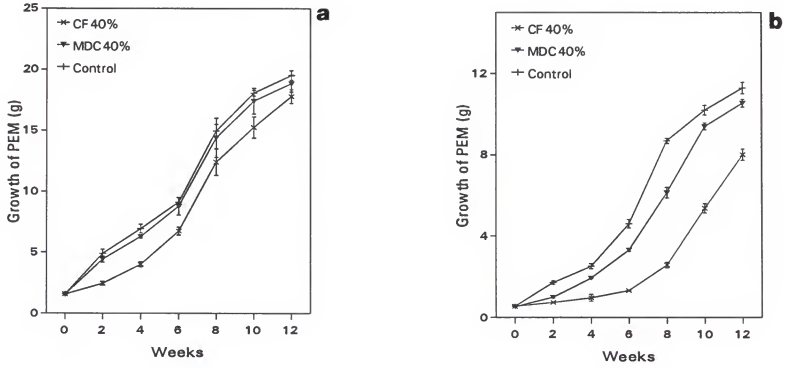

Fig. 4.2. Effect of 40% (v/v) culture filtrate on the growth of (a) 'Hindi' PEM and (b) 'Carabao' PEM.

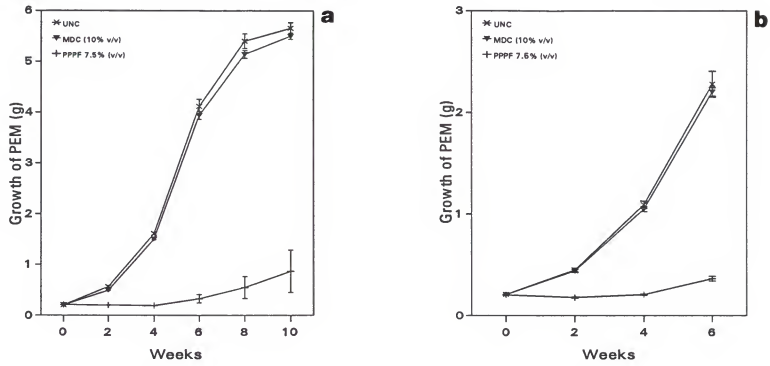

Fig. 4.3. Effect of PPPM 7.5% (v/v) on the growth of (a) 'Hindi' PEM and (b) 'Carabao' PEM.

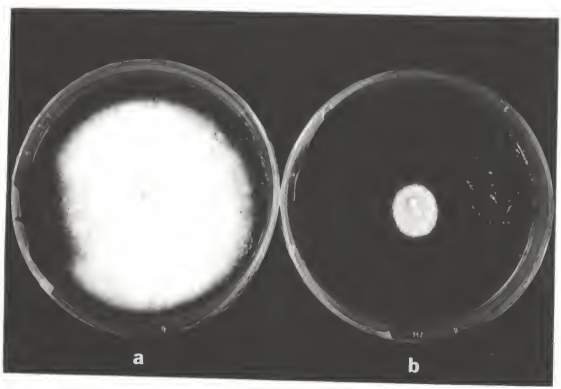

Fig. 4.4. Inhibition of mycelial growth in dual culture by (a) Control and (b) selected 'Hindi' line PRL III.

Table 4.3. Area of mycelial colony in dual culture with various lines of 'Hindi' and 'Carabao', seven days after dual culture.[1]

Treatment (Line)	HINDI[2]	CARABAO[2]
Control	39.28 ± 3.43a	41.47 ± 2.11a
PRL I	41.36 ± 3.29a	39.36 ± 1.15a
PRL II	33.54 ± 1.94b	36.18 ± 1.53b
PRL III	8.55 ± 1.44c	10.20 ± 1.15c
PRL V	8.58 ± 0.97c	10.82 ± 0.30c
PTR	8.59 ± 2.31c	8.87 ± 0.78c
Regression coefficient (r^2)	0.940	0.971

[1]Mean area in cm^2 ± standard error
[2]Means with same letter do not differ by Duncan's multiple range test at p=0.05

ANOVA

Source	DF	Sum of Squares	Mean Square	'F' value	Pr > F
'Hindi'					
Treatment	5	5347.132	1069.426	52.82	0.0001
Error	17	344.166	20.245		
Corrected Total	22	5691.298			
'Carabao'					
Treatment	5	5626.653	1125.331	23.25	0.0001
Error	23	1113.138	48.397		
Corrected Total	28	6739.791			

Table 4.4. Area of mycelial colony in conditioned medium containing 20% (w/v) of macerated cultures from various lines, 10 days after inoculation.[1]

Treatment (Line)	HINDI[2]	CARABAO[2]
Control	41.56 ± 1.13a	40.63 ± 0.61a
PRL I	39.98 ± 1.25a	39.86 ± 0.96a
PRL II	40.41 ± 0.99a	39.50 ± 0.59a
PRL III	40.76 ± 1.09a	40.06 ± 0.48a
PRL V	40.18 ± 0.64a	40.51 ± 0.56a
PTR	41.67 ± 1.18a	41.45 ± 1.24a
Regression Coefficient (r^2)	0.084	0.137

[1]Mean area in cm^2 ± Standard error
[2]Means with same letter do not differ by Duncan's multiple range test at p=0.05

ANOVA

Source	DF	Sum of Squares	Mean Square	'F' value	Pr > F
'Hindi'					
Treatment	5	12.661	2.532	0.44	0.8141
Error	24	137.227	5.718		
Corrected Total	29	149.888			
'Carabao'					
Treatment	5	11.791	2.358	0.76	0.5868
Error	24	74.387	3.099		
Corrected Total	29	86.178			

Conditioned medium experiment. Fungal growth was uniform in all the plates containing the macerated embryogenic cultures. No significant differences were observed between any of the selected lines and the unchallenged controls (Table 4.4). The mycelial colony almost covered the entire plate 10 days after inoculation. There were also no significant differences between the cultivars. The very low regression coefficients of 0.084 in 'Hindi' and 0.137 in 'Carabao' explains the absence of any effect of the macerated cultures on fungal growth.

4.4 Discussion

High concentrations of culture filtrate were toxic to mango embryogenic cultures. Resistance to the culture filtrate can be increased in embryogenic cultures by progressively increasing the concentration of culture filtrate during recurrent selection. Such increase in resistance after selection with culture filtrate has also been reported in peach (Hammerschlag, 1984) and alfalfa (Yu *et al.*, 1990). The use of crude culture filtrate as a selective agent for selecting *Verticillium* resistance in eggplant also has been reported by Rotino *et al.* (1990). It was observed that the embryogenic cultures darkened completely after the second challenge. The necrotic response may be due to the exudation of phenolic compounds (Litz *et al.*, 1982; 1984) or a hypersensitive defense reaction (Doke and Ohashi, 1988; Rogers *et al.*, 1988).

Embryogenic cultures followed a typical sigmoid pattern of growth in suspension as observed by Gavish *et al.* (1992) with *Citrus*. Significant repression of growth by the culture filtrate suggests that inhibitory compounds are produced by the fungus in broth culture. Litz *et al.* (1991) also observed such inhibition of growth in mango embryogenic cultures following cocultivation with culture filtrate

of *C. gloeosporioides.* Repression of growth has also been reported in alfalfa suspensions cocultured with culture filtrate of *Verticillium albo-atrum* (Frame *et al.,* 1991) and *Fusarium spp.* (Binarova *et al.,* 1990). The current study also illustrates the greater resistance of 'Hindi' than 'Carabao' to the culture filtrate, as 'Carabao' takes more time to recover after withdrawing the selection pressure. In addition, the phytotoxic metabolite also inhibited the growth of embryogenic cultures similar to that of culture filtrate. Partially purified toxins have been shown to be a useful selective agent for recovering *mal secco* resistance in *Citrus* (Nachmias *et al.,* 1977; Sesto *et al.,* 1990).

Dual culture studies with the selected lines showed increased mycelial growth inhibition with increasing number of cocultures. The current study indicates that at least three cycles of recurrent selection are necessary to induce significant levels of resistance in mango. Since mycelial growth was not suppressed in the conditioned medium (containing the macerated selected lines) studies, it is probable that the antifungal compound is extracellular in nature. Secretion of extracellular compounds, especially proteins, by embryogenic cell cultures has been reported by Ohashi and Matsuoka (1987), Esaka *et al.* (1990), Kragh *et al.* (1991), Gavish *et al.* (1991; 1992), Gentile *et al.* (1993) and von Arnold *et al.* (1995). In most cases the exact function of these extracellular secretions is unknown. However, De Jong *et al.* (1992), Zimmerman (1994), and Hendricks and de Vries (1995) have shown that certain proteins have to be correctly folded and targetted outside the cell wall in order to achieve *in vitro* somatic embryogenesis in carrot. Secretion of enzymes and metabolites into the extracellular space is a common phenomenon in bacterial, fungal and animal cells (Wink, 1994). Plants are known to secrete several metabolites into the apoplastic space (Fink *et al.* 1988), which may have a function in cell to cell

communication (Hendricks and de Vries, 1995) and defense proteins such as chitinases and glucanases are secreted into the extracellular matrix following a pathogen attack (Lamb *et al.*, 1992; Dixon *et al.*, 1994). Challenged embryogenic cells probably release these defense proteins into the culture medium as an analogous stress-induced response. Wink (1993) analyzed the spent medium from lupin and found that the composition of organic acids, amino acids and enzymes was very similar to that of vacuoles and suggest that the medium functions as a big extracellular vacuole.

Embryogenic cells of mango are actively dividing in suspension culture and are cytoplasmically rich cells. Several genes (~ 15,000 genes) are known to be expressed during embryogenesis (Goldberg *et al.*, 1994). These small embryogenic cells possibly do not have sufficient vacuolar space, where most of the mature proteins are targetted. This would necessitate the extracellular secretion of metabolites in suspension culltures.

In certain instances, especially under stress, enhanced secretion of pathogenesis related proteins such as chitinases and β-1,3-glucanases has been reported (Nishiwara and Hibi, 1991; Cabello *et al.*, 1994; Esaka *et al.*, 1994). It is possible that such enhanced release of pathogenesis-related proteins in the selected isogenic lines of 'Hindi' and 'Carabao' could confer anthracnose resistance, since a positive correlation between the enhanced release of these proteins and host resistance has been established in lemon (Gentile *et al.*, 1993) .

CHAPTER 5

EMBRYOGENIC MANGO CULTURES SELECTED FOR RESISTANCE TO CULTURE FILTRATE AND PHYTOTOXIC METABOLITE OF *COLLETOTRICHUM GLOEOSPORIOIDES* SHOW ENHANCED RELEASE OF ANTIFUNGAL PROTEINS. II. Chitinase and β-1,3-glucanase.

5.1.Introduction

During pathogen infection, plants are induced to express some natural defense responses. A number of polypeptides that combat pathogen infection are induced (Roby *et al.*,1990). As a result, disease is often the exception rather than the rule. Among the expressed polypeptides are antifungal hydrolases or pathogenesis-related proteins (PR proteins) that are expressed in greater quantities (Legrand *et al.*, 1987; Mauch *et al.*, 1988a).

Plants are known to produce chitinases (EC 3.2.1.14) and β-1,3-glucanases (EC 3.2.1.6) (Molano *et al.*, 1979; Pegg and Young, 1982; Boller *et al.*, 1983; Leah *et al.*, 1987 and Kragh *et al.*, 1991), but their function in plant metabolism is poorly understood (Boller, 1985; Fink *et al.*, 1988). In only two instances, viz., somatic embryogenesis in carrot (De Jong *et al.*, 1992) and sexual reproduction (Leung, 1992), has a specific role for chitinases been established. On the other hand, key components of cell walls of many of the plant pathogenic fungi, including *C. gloeosporioides*, are chitins and β-1,3-glucans (Wessels and Sietsma, 1981; Broekart *et al.*, 1989; Cornellissen and Melchers, 1993). These enzymes probably function in plants as a defense mechanism (Bol *et al.*, 1990; Bowles, 1990; Dixon and Harrison, 1990; Collinge *et al.*, 1993). Other proteins such as ribosome-inactivating proteins (RIPs) (Roberts and Seletrennikoff, 1988; Endo *et al.*, 1988; Stirpe and Hughes 1989; Leah *et al.*, 1991; Logemann *et al.*, 1992), stress-inducible

Stirpe and Hughes 1989; Leah *et al.*, 1991; Logemann *et al.*, 1992), stress-inducible proteins (Carr and Kleissig, 1989), and lectins (Broekaert *et al.*, 1989; Chrispeels and Raikhel, 1991) are also implicated as part of the plant defense against pathogens.

Plant chitinases and β-1,3-glucanases occur in several isoforms, differing in physical and molecular properties, enzyme activity (Melchers *et al.*, 1993a), cellular compartmentalization, localization and antifungal properties (Cabello *et al.*, 1994). Both of these enzymes are encoded by a small multigene family (Linthorst, 1991). This isoenzyme multiplicity enables plant cells to deploy one form or other in a tissue-specific or stimulus-specific manner (Mardis-Pinheiro *et al.*, 1991). Chitinases and/or β-1,3-glucanases are induced not only during pathogen infection, but also in response to other stress factors such as ethylene and wounding (Boller *et al.*, 1983; Cabello *et al.*, 1994), ozone (Schraudner *et al.*, 1992), salicylic acid (Williams and Leung, 1993) salt adaptation (Esaka *et al.*, 1994) and soluble silicon (Cherif *et al.*, 1994).

Extracellular chitinases are predominantly acidic (Graham and Sticklen, 1994) and have been detected in intercellular washing fluids in intact plants (Kauffman *et al.*, 1987; Legrand *et al.*, 1987; Kombrink *et al.*, 1988) and as a secreted protein in the medium of suspension cultures (Esaka *et al.*, 1990; 1994; Gentile *et al.*, 1993). Most of the well characterized extracellular β-1,3-glucanases are also acidic (Kragh *et al.*, 1991; Flach *et al.*, 1993). It is widely believed that these extracellular forms of chitinase and β-1,3-glucanases are the first line of defense deployed by plant cells (Fink *et al.*, 1988; Mauch and Staehelin 1989; Krebs and Grumet 1993).

As a part of this study, it was found that embryogenic suspension cultures of mango selected against culture filtrate or the phytotoxic metabolite from *C. gloeosporioides* secrete extracellular antifungal compounds. In this study, characterization of these extracellular proteins that are believed to confer resistance to the fungus in the embryogenic cultures are presented.

5.2 Materials and Methods

Extraction of extracellular proteins and quantitation. Embryogenic cultures (2.5g) of the resistant lines and the unchallenged controls that were identified in both cultivars were grown in 40 ml suspension culture medium at 120 rpm under low light conditions. On the 6th or 12th day after culture (DAC), the suspension cultures were subcultured and the spent medium was collected. The cellular debris was removed by low speed centrifugation in a clinical centrifuge (2,000 rpm) for 5 min. To the supernatant two and a half volumes of cold ethanol (95%) was added and the proteins were precipitated overnight at -20 °C (deVries *et al.*, 1988). The proteins were collected by centrifugation at 4°C (10,000 rpm for 10 min). The protein pellets were resuspended in 1 ml of 20 mM Tris-HCl and quantified using the Bradford method (Bradford, 1976) with bovine serum albumin as a standard.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of extracellular proteins. SDS-PAGE was performed in 12% (w/v) polyacrylamide gradient gels (160 x 180 x 1.5 mm) containing 0.1% (w/v) SDS (Laemmli, 1970). The stacking gel consisted of 5% (w/v) polyacrylamide and 0.1% (w/v) SDS. 30 μg

of proteins from each line was analysed in a LKB 2001 vertical electrophoresis system at 100mA constant current. The samples were diluted with 20 µl of loading buffer containing 400 mg SDS, 2 ml glycerol, 1 ml mercaptoethanol, 4 mg bromophenol blue and 0.125 M Tris-HCl in a total volume of 10 ml and boiled for 10 min before loading. Electrophoresis was carried out in Tris -Glycine- SDS buffer at pH 8.9 (Davis, 1964). Protein standards (Sigma) were used as molecular weight markers. After electrophoresis the gels were stained with either Coomassie Brilliant Blue R 250 or silver staining kit (Sigma).

Extraction and analysis of total soluble proteins. Soluble proteins were extracted as described earlier (Krochko and Bewley, 1990) with slight modifications. Embryogenic callus and mature somatic embryos from various lines were ground to a fine powder in liquid nitrogen. The powder was then homogenized in a low salt buffer [200 mM sodium chloride in 25 mM potassium phosphate, pH 7.0 containing 1 mM phenylmethylsulfonylfluoride (PMSF) and leupeptin] and the supernatant was collected after centrifugation at 10,000 rpm for 10 min. The remaining pellet was resuspended in a high salt buffer (1 M sodium chloride in 25 mM potassium phosphate, pH 7.0 and 1 mM PMSF) and the supernatant was collected as before. Both the supernatants were mixed together and the proteins were precipitated with cold ethanol or acetone (2 volumes) overnight at 4°C. The precipitated proteins were pelleted after centrifugation and resuspended in 20 mM Tris-HCl and stored at -20°C until used.

Native PAGE and detection of chitinase activity. Polyacrylamide resolving gels (15%, 160x180x1.5 mm) were prepared by casting a mixture of 15.0 ml of 30% polyacrylamide mix (acrylamide/Bis 30:0.8), 3.75 ml 3.0 M Tris-HCl (pH 8.8), 600 µl of 10% ammonium persulfate, 9.75 ml sterile deionized water and 20 µl of TEMED. The stacking gel included 2.5 ml of 30% acrylamide mix, 5.0 ml of 500 mM Tris-HCl (pH 6.8), 400 µl of 10% ammonium persulfate, 11.5 ml of sterile deionized water and 20 µl of TEMED. Protein samples were diluted with equal volumes of loading buffer (Laemmli, 1970) lacking SDS and mercaptoethanol. Electrophoresis was performed at 10° C at 100 mA constant current in an LKB 2001 vertical electrophoresis unit. After electrophoresis, chitinase activity was detected using an overlay gel containing glycol chitin (Trudel and Asselin, 1989; Pan *et al.*, 1991). The gels were incubated in 150 mM sodium acetate buffer (200 ml/gel) at pH 5.0 for 10 min with gentle shaking. They were then placed on a clean glass plate and covered with a 7.5% polyacrylamide overlay gel (160 x 180 x 0.75 mm), containing 0.01% (w/v) glycol chitin in 100 mM sodium acetate buffer (pH 5.0). Glycol chitin was synthesized by acetylation of glycol chitioson (Molano *et al.*, 1979). After eliminating all of the air bubbles and any liquid between the gels, they were incubated at 37°C for 2 h under moist conditions. The overlay gel was separated carefully and then incubated in freshly prepared 0.01% (w/v) fluorescent brightener 28 (= Calcofluor White M2R) in 500 mM Tris-HCl, pH 8.9 at room temperature with gentle shaking for 10 min. The brightener solution was discarded

and the overlay gel was stored in sterile deionized water for 2 h at room temperature. Chitinase isozymes were visualized as cleared zones or lytic bands when the gel was placed over a UV transilluminator. The gel was photographed with Polaroid type 55 film with UV haze and 02 orange filters. The exposure time varied from 90s to 120s at *f* 4.7.

Detection of chitinase activity after SDS-PAGE. SDS-PAGE was performed as described earlier, but the resolving gels were incorporated with 0.01% (w/v) glycol chitin. After electrophoresis, the gels were incubated for 2 h at 37°C with gentle shaking in 100 mM sodium acetate buffer (pH 5.0) containing 1% (v/v) Triton X-100. Gels were then stained with 0.01% (w/v) fluorescent brightener 28 in 500 mM Tris-HCl (pH 8.9) and destained in sterile deionized water. Chitinase isozymes were identified by reading the gels over a UV transilluminator and photographed. In both of the above experiments standard chitinases from *Streptomyces griesius* and *Serratia marcesens* (Sigma) were used as controls.

Detection of glucanase after native PAGE (Pan *et al.*, 1991). Native PAGE was performed as described earlier (Davis, 1964). After electrophoresis, the gels were washed with sterile deionized water and incubated in 50 mM sodium acetate (pH 5.0) for 5 min. Meanwhile, laminarin (500 mg) was dissolved in 40 ml of sterile deionized water by warming in a water bath and after cooling to room temperature, mixed with 40 ml of 50 mM sodium acetate (pH 5.0). The gels were removed from sodium acetate buffer and incubated in laminarin solution at 40°C for 30 min. After impregnation with laminarin, the gels were fixed in a methanol: water: acetic acid

(5:5:2, 200ml/gel) buffer for 5 min. After thorough washing with sterile deionized water, the gels were stained with 300 mg of 2,3,5-triphenyltetrazolium chloride in 200 ml of 1 M sodium hydroxide in a boiling water bath. Glucanase isozymes appeared as red bands after approximately 10-12 min. Laminarinases from *Penicillium* spp. and mollusk (Sigma) served as standards. The gels were photographed immediately and stained with Coomassie Brilliant Blue R 250, as the gels began to expand and tended to break off. Glucanase isozymes could be identified even after Coomassie Blue staining as purple bands, while the other proteins stained blue.

5.3 Results

The amount of extracellular proteins precipitated after 6 and 12 days from cultures of the various putatively phytotoxin resistant lines of both cultivars are presented in Table 5.1. In general, the amount of proteins precipitated after 12 days was much higher than after 6 days. There were no significant differences between unchallenged controls and selected putatively resistant lines in extracellular protein content 6 days after culture; however, significant differences were observed between selected lines and unchallenged controls 12 days after culture. The unchallenged controls of 'Carabao' yielded the lowest amount of protein both 6 and 12 days after culture (18.00 and 20.20 μg/ml, respectively), while the lines PRL III of 'Hindi' and PRL V of 'Carabao' yielded the highest amount of proteins 12 days after culture (38.33 and 37.77 μg/ml, respectively).

SDS-PAGE also revealed differences in protein profiles between recurrently selected lines and unchallenged controls. The protein profile also differed between 'Carabao' and 'Hindi'. Protein profiles were not clear in the extracellular proteins

Table 5.1. Extracellular protein content in the spent medium of various lines of 'Hindi' and 'Carabao.'[1]

LINE	'HINDI'		'CARABAO'	
	6 DAC	12 DAC	6 DAC	12 DAC
Control	18.33 ± 0.17a	20.47 ± 1.05a	18.00a	20.20 ± 0.10a
PRL I	18.37 ± 0.07a	20.43 ± 0.82a	19.00a	21.27 ± 0.15a
PRL II	18.33 ± 1.17a	27.33 ± 0.33b	18.93 ± 0.07a	26.33 ± 0.17b
PRL III	18.33 ± 0.17a	38.33 ± 0.17c	19.10 ± 0.10a	29.00 ± 0.37b
PRL V	18.67 ± 0.17a	30.60 ± 0.38b	19.00a	37.77 ± 0.54c
PTR	18.67 ± 0.17a	30.50 ± 0.29b	18.93 ± 0.07a	29.50 ± 0.88b
Regression Coefficient (r^2)	0.331	0.982	0.268	0.996

[1]Mean protein content ± standard error
[2]Means with same letter do not differ by Duncan's multiple range test at p=0.05

ANOVA 'Hindi'

Source	DF	Sum of Squares	Mean Square	'F' value	Pr > F
6 DAC					
Treatment	5	0.425	0.085	1.19	0.3718
Error	12	0.860	0.071		
Corrected Total	17	1.285			
12 DAC					
Treatment	5	702.658	140.534	130.46	0.0001
Error	12	12.927	1.077		
Corrected Total	17	715.585			

contd. on next page

ANOVA 'Carabao'

Source	DF	Sum of Squares	Mean Square	'F' value	Pr > F
6 DAC					
Treatment	5	2.523	0.505	53.42	0.0001
Error	12	0.113	0.009		
Corrected Total	17	2.636			
12 DAC					
Treatment	5	598.298	19.660	690.34	0.0001
Error	12	2.080	0.1731		
Corrected Total	17	600.378			

precipitated 6 days after culture. Hence, proteins precipitated after 12 days after culture alone were studied in the subsequent analyses.

In 'Hindi,' 2 prominent bands of 31 and 33 kD was produced in all of the lines; however, expression of the 31 kD band increased in proportion with the number of challenges. The phytotoxic metabolite resistant lines also expressed this band strongly. In addition, a new band of approximately 45 kD was expressed by the most resistant lines (Fig. 5.1). This band co-migrated with one of the bands produced by commercial chitinase from *Serratia marcescens* (Sigma). The extracellular protein profile of 'Carabao' was different from that of 'Hindi.' The unchallenged controls exhibited a strong band of approximately 27 kD, while this was weakly expressed by the putatively tolerant lines. On the other hand, a band of approximately 32 kD was expressed strongly by the putatively resistant lines in

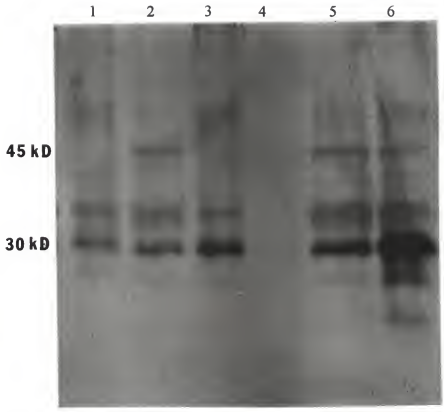

Fig. 5.1. SDS-PAGE separation of extracellular proteins from the spent medium of various lines of 'Hindi.' [Lanes. 1. Control, 2. PRL III, 3. PRL II, 4. Standard Chitinase , 5. PRL V, 6. PTR]

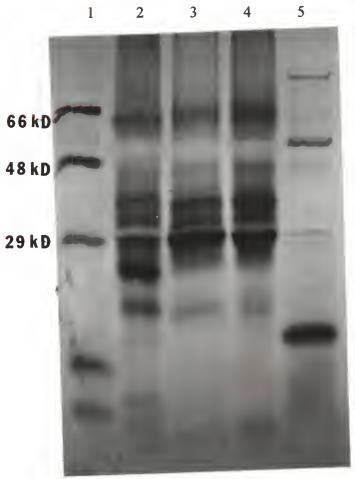

Fig. 5.2. SDS-PAGE separation of extracellular proteins from the spent medium of 'Carabao.' [Lanes. 1. Molecular Weight markers, 2. Control, 3. PRL III, 4. PRL V, 5.Standard Chitinase]

Table 5.2. Total soluble protein content from the PEM and mature somatic embryos of various lines of 'Hindi' and 'Carabao.'[1]

LINES	PEM		Mature Somatic Embryos	
	'Hindi'[2]	'Carabao'[2]	'Hindi'[2]	'Carabao'[2]
Control	24.17 ± 0.66	24.57 ± 0.66	20.933 ± 0.811	20.367 ± 0.845
PRL III	24.47 ± 0.77	25.82 ± 1.06	20.200 ± 0.721	19.523 ± 0.751
PRL V	25.83 ± 0.58	24.30 ± 0.61	20.761 ± 0.949	20.133 ± 1.048
PTR	24.07 ± 1.21	23.60 ± 1.25		

[1] Values are mean ± standard error.
[2] Means within a column do not differ significantly at p = 0.05.

ANOVA
'Hindi'
PEM

Source	DF	Sum of Squares	Mean Square	'F' value	Pr > F
Treatment	3	6.020	2.007	0.94	0.4642
Error	8	17.027	2.218		
Corrected Total	11	23.047			
Mature Somatic Embryo					
Treatment	2	0.887	0.443	0.21	0.8138
Error	6	12.473	2.079		
Corrected Total	8	13.360			
'Carabao'					
PEM					
Treatment	3	7.829	2.610	1.00	0.4410
Error	8	20.873	2.609		
Corrected Total	11	28.702			
Mature Somatic Embryo					
Treatment	2	1.109	0.554	0.23	0.7988
Error	6	14.260	2.377		
Corrected Total	8	15.369			

comparison with the unchallenged control. In addition, a 45 kD band was also expressed, although weakly, by the lines PRL V and PRT (Fig. 5.2).

The total soluble proteins from the embryogenic cultures and mature somatic embryos of various lines of 'Hindi' and 'Carabao' are presented in Table 5.2. There were no significant differences in protein content between unchallenged controls and recurrently selected putatively resistant lines in both cultivars. Similarly, there were no major differences between the controls and selected lines with respect to protein profile after SDS-PAGE separation. The total soluble proteins from the embryogenic cultures showed 2 bands of approximately 30 and 35 kD, while the total soluble proteins from mature somatic embryos revealed 3 bands of 30, 33 and 35 kD.

Chitinase isozymes were clearly visible in the overlay gels following native PAGE. The extracellular proteins of all selected lines and controls of both cultivars exhibited chitinase activity. In 'Hindi', the unchallenged controls exhibited only 2 isozymes of chitinase of approximately 30 and 32 kD molecular weight. The selected putatively resistant lines, PRL III, PRL V and PTR, exhibited these isozymes in a greater quantity, as visualised by the intensity of hydrolyzation. In addition, all of the three resistant lines exhibited an additional isozyme of approximately 45 kD. This isozyme seems to have much greater hydrolytic activity than the other two isozymes, since the lytic zones appeared to be much darker than the other two isozymes (Fig. 5.3a). In 'Carabao' all of the lines exhibited an isozyme of approximately 27 kD; however, this isozyme exhibited a stronger activity in PRL V and in the putatively toxic fraction tolerant line. All of the lines exhibited weak 33 kD chitinase activity. The lines PRL V and PTR showed a weak lytic zone at 45 kD. Line PRL III exhibited a unique, but weak band of approximately 25 kD, which was

not present in any other line (Fig. 5.3b). Together the results show that recurrent selection against the culture filtrate or the phytotoxic metabolite results in either enhanced activity of chitinase isozymes or induction of new isozymes of chitinases in the extracellular proteins.

Chitinase activity could be detected after SDS-PAGE, indicating the hardy nature of the enzyme; however, renaturation of the enzyme was not consistently uniform, often resulting in varying expression of chitinase activity. There were 3 isozymes in the selected resistant lines of 'Hindi' separating at 30, 33 and 45 kD, while only two were present in the unchallenged control. This confirms that the chitinase isozymes exhibited by these lines are monomers. In 'Carabao' the number of bands showing lytic activity ranged from 1 in the unchallenged control to 4 in PRL V, in accordance with their separation in native PAGE.

High glucanase activity could be seen in the extracellular proteins of the putatively resistant lines of 'Hindi,' in comparison with unchallenged control. The line PRL III showed the greatest activity of glucanase as visualised by the quickly appearing dark red band. The band in this line appeared even before the standard. There was only one isozyme of glucanase (approximately 30 kD). In contrast, glucanase activity in the extracellular protein fractions of 'Carabao' was very weak in all the lines. While all the lines showed only one band, PRL V of 'Carabao' showed an additional band of lower molecular weight (approximately 25 kD).

The results of this study indicate that challenge inoculation with culture filtrate or with the putatively phytotoxic metabolite from *C. gloeosporioides* results in the enhanced secretion of extracellular proteins by these lines. This secretion increases in proportion with increasing numbers of challenges. Increased activity of

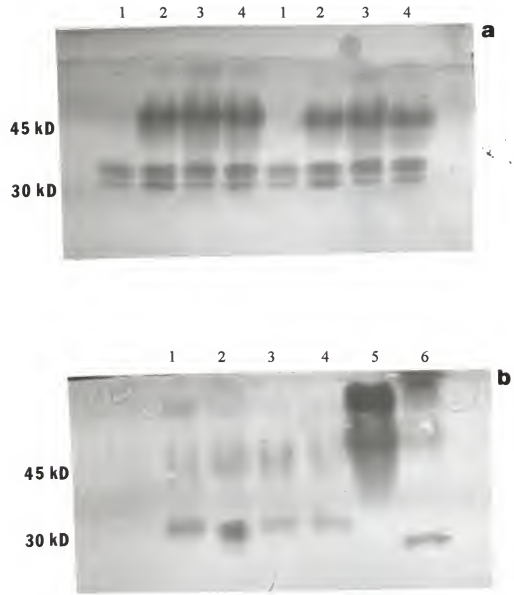

Fig. 5.3. Chitinase activity in the extracellular protein of (a) 'Hindi' and (b) 'Carabao.' [Lanes. 1. Control, 2. PRL III, 3. PRL V, 4. PTR, 5. & 6. Chitinase Standards.]

two pathogenesis-related proteins viz., chitinase and β-1,3-glucanase, was observed in the extracellular proteins secreted by recurrently selected lines. On the contrary, no significant differences could be observed in the total soluble proteins either from embryogenic cultures or from mature somatic embryos, suggesting that the fungal mycelial growth inhibition by these selected lines is probably due to the extracellular proteins.

5.4 Discussion

Plants are continuously exposed to pathogens in their natural environment, and are believed to have co-evolved with their pathogens (Lamb, 1994). Since plants, unlike animals, lack the ability to produce antibodies, they have evolved their own distinct defense mechanism (Walbot, 1985). In contrast to animal cells, a plant cell is capable of defending itself by a combination of constitutive and induced genes that are expressed specifically following a pathogen attack (Lamb *et al.*, 1989; Ryals *et al.*, 1992; 1994). As a result, susceptibility to the pathogen is the exception and resistance is the rule (Staskawicz *et al.*, 1995). Intact plants or plant tissues resist pathogen attack in a variety of ways often correlated with a hypersensitive response at the infection site (Keen *et al.*, 1993; Melchers *et al.*, 1993a). Oxidative burst, ion fluxes, changes in the cell wall, production of phytoalexins and induction of pathogenesis-related proteins, such as the chitinases, glucanases and hydrolases are some of the well characterized events leading to the hypersensitive response in plant tissues.

Embryogenic cultures consisting of proembryonic masses (PEM), grown in suspension cultures demonstrate some of the defense responses, e.g., the enhanced secretion of pathogenesis related proteins including chitinases and glucanases into

the culture medium. Enhanced secretion of chitinase and glucanase was observed in nucellar cultures selected for *'mal secco'* resistance (Gentile *et al.,* 1993; Deng *et al.,* 1995). In addition, extracellular secretion of pathogenesis-related proteins has been shown to be induced by pathogens and elicitors in tobacco (Antoniw *et al.,* 1981; Ohashi and Matsuoka, 1987).

Embryogenic suspension cultures of mango are subjected to stress during recurrent selections and hence the secretion of chitinases and β-1,3-glucanases could be pathogenesis-related, since the culture filtrate or the purified fraction from the fungus were used as the selective agent. Such enhanced extracellular secretion of chitinases and glucanases has been observed following salt stress in winged bean (Esaka *et al.,* 1994), culture stress in Virginia creeper (*Parthenocissus quinquefolia*) (Flach *et al.,* 1993), lupin (*Lupinus albus* L) (Wink *et al.,* 1992) or after treatment with fungal mycelium in carrot (Kurosaki *et al.,* 1988) and *Picea abies* (Sauter and Hager, 1989). In addition, Wink (1994) suggests that secretion of these hydrolases after stress in liquid cultures could be an analogus response to the extracellular secretion of these enzymes by intact plants following pathogen attack or stress. In all these studies the chitinases and β-1,3- glucanases that were characterized were acidic, corroborating the present results.

In contrast, Kragh *et al.* (1991) purified three basic chitinases and one basic glucanase from the spent medium of suspension cultured barley cells. Basic chitinases and glucanases are generally localized intracellularly (Melchers *et al.,* 1993a, b). Such intracellular chitinases and glucanases have been isolated and characterized from roots following mycorrhizal or fungal infection in *Picea abies* (Sauter and Hager, 1989), *Eucalyptus globulus* (Albrecht *et al.,* 1994) and cucumber (Cherif *et al.,* 1994). In addition, aerial plant parts such as leaves and

stems accumulate the basic isoforms of either or both of these proteins intracellularly in response to other known inducers such as ethylene, elicitors, organic molecules, salts, salicylic acid and virus (Flach *et al.,* 1992; Cornellissen and Melchers 1993; Graham and Sticklen, 1994). In the present study only weak activity of chitinase and glucanase was seen in the soluble intracellular proteins extracted from mature somatic embryos, indicating that basic isoforms of these enzymes are probably not involved in anthracnose resistance.

Conflicting reports exist about the antifungal activity of chitinases and glucanases. While it has been well documented with a number of species that several isoforms of these proteins are induced in fungal infection, only a few isozymes have been shown to have *in vitro* antifungal activities (Flach *et al.,* 1992; Cornellissen and Melchers, 1993; Graham and Sticklen, 1994). There is strong agreement among published reports that the two enzymes act synergistically in plant defense. The extracellular isozymes, which are acidic, are implicated in the early line of defense in plants (Collinge *et al.,* 1993; Graham and Sticklen, 1994). They can act directly by blocking the growth of fungal hyphae or indirectly by signalling the release of other mechanisms such as elicitors in the host (Roby *et al.,* 1987; Kurosaki *et al.,* 1988; Barber *et al.,* 1989). In the current study, fungal growth inhibition was caused by extracellular proteins as the conditioned medium containing the total soluble proteins extracted from resistant lines did not show any fungal inhibition, which is in agreement with the above findings. Since a new isozyme as well as enhanced quantities of other isozymes of chitinase and glucanase are secreted extracellularly into the medium by the resistant lines, it is suggested that these proteins also confer antifungal activity in the selected lines. In contrast, Melchers *et al.* (1993a) and Joosten *et al.,* (1995) have shown that the extracellular isoforms are not antifungal,

either alone or in concert, in tobacco and tomato, respectively. Together the evidences suggest that only certain isoforms of these enzymes are antifungal and these are induced specifically following pathogen infection.

Genes encoding chitinases and glucanases have been identified and cloned from several plant species (Graham and Sticklen, 1994). Transgenic tobacco plants constitutively expressing chitinase and/or β-1,3-glucanase genes showed enhanced resistance to *Rhizoctonia solani* (Broglie *et al.*, 1991), *Cercospora nicotiana* (Neuhaus *et al.*, 1991), *Phytophthora infestans* (Melchers *et al.*, 1993 a,b). Overexpressing these enzymes in mango using existing transformation protocols (Mathews *et al.*, 1992) is an attractive possibility to enhance tolerance of anthracnose and other fungal diseases; however, it would be much more effective to use the systemic response, since it is the defense response exhibited by the plant in a plant-pathogen interaction (Bol *et al.*, 1990; Bowles, 1990). This response, now better known as systemic acquired resistance (SAR), is becoming a subject of increasing inquiry. While most of the defense responses are expressed locally at the site of infection only, SAR is expressed in the whole plant. Several SAR genes or gene products have anti-microbial activity or are closely related to classes of anti-microbial proteins (Kessmann *et al.*, 1994). Among these genes are those encoding certain, mostly acidic isoforms of chitinases and β-1,3-glucanases (Ward *et al.*, 1991; Meins *et al.*, 1992). In the current study, induction and enhanced expression of chitinases and glucanase in the recurrently selected resistant mango lines could be a SAR response, as these are acidic extracellular isoforms and are antifungal.

More emphasis has been placed on discovery of new resistance genes and overexpressing these genes using molecular techniques than on using the resistance potential already existing in plants. Recent evidence shows the effectiveness of SAR

in the laboratory as well as in the field (Kessmann *et al.*, 1994). Since SAR could also result in a broad-spectrum disease resistance (Alexander *et al.*, 1993) it would be a better option than overexpression of antifungal hydrolase; however, all the studies presently available involve herbaceous crops. It remains to be seen how far SAR will be effective in a perennial crop like mango.

CHAPTER 6
EMBRYOGENIC MANGO CULTURES SELECTED FOR RESISTANCE AGAINST THE CULTURE FILTRATE OF *COLLETOTRICHUM GLOEOSPORIOIDES* SHOW VARIATION IN RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) MARKERS

6.1 Introduction

Using conventional breeding to introduce important horticultural traits such as disease resistance into existing and newly developed mango (*Mangifera indica* L.) cultivars has been futile due to long juvenile periods, extremely poor fruit set and heterozygosity (Mathews and Litz, 1992; Iyer and Degani, in press). *In vitro* selection to generate genotypes that show enhanced disease resistance, salt tolerance or herbicide tolerance has been recognized as an important tool in perennial fruit breeding (Hammerschlag, 1992). This approach is of particular advantage with perennial crops including mango, as it is possible to screen a large number of embryogenic cells against a selection factor under controlled conditions (Hammerschlag, 1992). Recovery of regenerants having enhanced disease resistance using *in vitro* selection has been achieved in few perennial species (van den Bulk, 1991; Hammerschlag, 1992). Among these, the two most comprehensively documented *in vitro* selection studies have involved peach (*Prunus persica* L.) and lemon (*Citrus limon* L.). Enhanced tolerance of peach for bacterial leaf spot disease, caused by *Xanthomonas campestris* pv. *pruni*, was reported using embryogenic cultures derived from immature zygotic embryos (Hammerschlag, 1988, 1990), while a *mal secco*-tolerant line was recovered from embryogenic nucellar cultures of lemon (Gentile *et al.*, 1992a, b).

Another approach seldom employed due to its high cost has involved screening large numbers of *in vitro* propagated plants in the field for heightened disease resistance or tolerance. Although disease-resistant regenerants have been reported for banana (*Musa* spp.), e.g., resistance to Panama disease caused by *Fusarium oxysporium* f. sp. *cubense* race 4 (Hwang and Ko, 1987; Hwang, 1990), similar studies with other perennial species are precluded because of the great expense involved. Such breeding methods with emphasis on phenotypic assay alone are notoriously difficult in perennial species, since most of the important horticultural traits involve quantitative or multigenic inheritance (Paterson *et al.*, 1988)

Molecular markers have been shown to be effective tools for enhancing breeding efforts in annual crops and have better practical value in perennial crops (Moore and Janick, 1983) since they overcome major environmental effects. Protein markers such as isozymes have been used in perennial crops like *Citrus* (Ashari *et al.*, 1988; Moore and Castle, 1988) grapes (*Vitis spp.*) (Parfitt and Arulsekar, 1989), lychee (*Litchi sinensis* L.) (Degani *et al.*, 1995) mango (Degani *et al.*, 1990, 1992, 1993), *Musa* spp. (Jarret and Litz, 1986a, b, c) and stone fruits (Arulsekar *et al.*, 1985; 1986a, b; Arulsekar and Parfitt, 1986), but only to construct partial linkage maps or to establish phenotypic correlations. Isozymes have also been used to evaluate F1 progeny or to distinguish the zygotic seedling from the nucellar seedlings in the progeny of polyembryonic *Citrus* cultivars (Torres *et al.*, 1982) and mango (Schnell and Knight, 1992). When restriction fragment length polymorphism (RFLP) based genetic mapping (Botstein *et al.*, 1980) was applied to plant breeding, the utility of DNA markers became apparent (Tanksley *et al.*, 1989). It was possible to locate several genes of agronomic interest within the context of an existing RFLP

map (Rafalsky and Tingey, 1993). Variations of this technique, i.e., variable number of tandem repeats (VNTRs) and minisatellites have also been used either alone or in conjunction with RFLPs (Bachmann, 1992). However, RFLPs are restricted to genomic regions with low or single copy sequences (Michelmore *et al.*, 1991). These methods have been effectively used for cultivar identification (Moore and Durham, 1992).

More recently, polymerase chain reaction (PCR) based methods such as random amplified polymorphic DNA (RAPD) have become popular for identifying molecular markers due to the speed and simplicity of the procedure in comparison with RFLPs (Tulseiram *et al.*, 1992). RAPD marker system is based on the PCR amplification of random DNA segments in the genome using primers (usually 10-mers) of arbitrary nucleotide sequence (Williams *et al.*, 1990). Allelic variation among individuals is detected as the presence or absence of the amplification product, visualized as a band after PCR and electrophoresis (Welsh and McCleland, 1990). Moreover, RAPD requires very low quantities of genomic DNA for amplification and involves no radioactivity (Michelmore *et al.*, 1991; Rafalsky and Tingey, 1993). Analysis of variation at the nuclear genome level using RAPDs has advantages over RFLPs, as a single primer produces several loci, covering a larger portion of the genome (Tulseiram *et al.*, 1992). RAPDs have been used to determine genetic relationships in *Annona* spp. (Ronning *et al.*, 1995a), cacao (*Theobroma cacao* L.) (Wilde *et al.*, 1992; Ronning *et al.*, 1995b) and papaya (Stiles *et al.*, 1993). In *Mangifera* spp., RAPDs have been used to determine phylogenetic relationships (Schnell and Knight, 1993) and also for cultivar identification in mango (Schnell *et al.*, 1995). RAPD markers have also been useful for confirming somatic hybrids following protoplast fusion between *Citrus* spp. (Deng *et al.*, 1995) and

between *Solanum* spp. (Rasmussen and Rasmussen, 1995). The present study describes the use of RAPDs to demonstrate markers for *in vitro* induced variation in mango embryogenic cultures following recurrent selection with the culture filtrate containing the phytotoxic metabolite produced by *C. gloeosporioides*.

6.2 Materials and Methods

Two mango cultivars, 'Carabao' and 'Hindi' were used in this study. 'Carabao,' a polyembryonic cultivar from the Philippines was available as specimen trees in the germplasm collection of the USDA, ARS Subtropical Horticultural Research Station, Miami, FL. 'Hindi' a polyembryonic cultivar from Egypt was available as specimen trees in the germplasm collection of the University of Florida, Tropical Research and Education Center, Homestead FL.

Embryogenic cultures and establishment of putatively tolerant lines. [Refer to Chapter 4, Materials and Methods, page 38.]

Proembryonic masses were subjected to recurrent selection with the culture filtrate of *C.gloeosporioides* and resistant lines were established of both cultivars and designated as putatively resistant lines (PRL) (see Chapter 4). Although four lines were established in each cultivar only the most resistant lines, PRL III and PRL V, were used in this study besides the unchallenged control. Samples removed from suspension cultures of these lines were used for DNA extraction. DNA was also extracted from leaves of field-grown trees of the two cultivars.

DNA Extraction. New leaves of 'Carabao' and 'Hindi' which were pinkish to copper brown in color were collected from the germplasm collections. DNA from leaves as well as from proembryonic masses was extracted using the hexadecyltrimethylammonium bromide (CTAB) method described by Doyle and

Doyle (1990). Three grams of fresh tissue were ground to a fine powder in liquid nitrogen. Grinding was continued with the addition of 22.5 ml of prewarmed (60°C) extraction buffer, which consisted of 4% (w/v) CTAB, 1.4 M NaCl, 0.1% (v/v) B-mercaptoethanol, 20 mM EDTA, 100 mM tris HCl (pH 8.0) and 1% (w/v) polyvinylpolypyrrolidone (PVPP). The homogenate was incubated at 60°C for 30 minutes and extracted with chloroform:isoamyl alcohol (24:1). The total nucleic acids were precipitated in cold isopropanol. The extraction and precipitation was repeated once. The nucleic acids were digested with RNAse A and DNA was precipitated and resuspended in sterile deionized water. The concentration of DNA was determined spectrophotometrically.

DNA Amplification. Amplification was carried out according to the protocol of Williams *et al.* (1990) and modified by Schnell *et al.* (1995). Primers from the primer kits A and F (Operon Technologies, Almeda, CA) were used in the amplification process. The sequences of the primers used in the study are presented in Table 6.1. The reactions contained 50 mM Tris HCl (pH 8.0), 100 mM NaCl, 0.1 mM EDTA, 5 mM DTT, 3.0 mM $MgCl_2$, 100 µM each of dATP, dCTP, dTTP and dGTP, 0.3 µM primer, 0.5 µl Taq DNA Polymerase (Promega Corporation, Madison, WI) and 25 ng genomic DNA in a total volume of 25 µl. Reactions were run for 45 cycles at 92°C for 1 min 35°C for 1 min and 72°C for 2 min in a DNA thermocycler 480 (Perkin-Elmer, Norwalk, CT). After amplification, 4 µl of bromophenol based loading buffer was added to the reaction mixture as the tracking dye. A 9.0 µl aliquot was loaded onto a 1.4% agarose gel and electrophoresed at 4.4 V/cm in tris-borate - EDTA buffer. The gels were stained with ethidium bromide and the amplification products were visualised under a UV transilluminator and identified with reference to size of base pairs. A 100 bp ladder (Gibco-BRL) served

Table 6.1. Primers and their sequences used in the study.

Primer #	5' → 3'	Primer #	5' → 3'
OPA 7	GAAACGGGTG	OPF 6	GGGAATTCGG
OPA 8	GTGACGTAGG	OPF 7	CCGATATCCC
OPA 10	GTGATCGCAG	OPF 12	ACGGTACCAG
OPA 11	CAATCGCCGT	OPF 13	GGCTGCAGAA
OPA 13	CAGCACCCAC	OPF 15	CCAGTACTCC
OPA 18	AGGTGACCGT	OPF 16	GGAGTACTGG
OPA 19	CAAACGTCGG	OPF 19	CCTCTAGACC
OPA 20	GTTGCGATCC		

as the size marker. Each amplification was performed at least twice. Data were recorded as discrete variables, with 1 representing the presence of a marker and 0 representing its absence.

6.3 Results

A total of 68 bands, identified as markers, were generated by the 15 primers. Of the 68 markers, 42 were produced by all of the 8 lines tested and 26 markers were either present additionally in the selected lines in comparison with the controls or absent. The numbers of markers (= loci) generated by the primers included 1 in OPA 8 to 8 in both OPA 11 and OPF 13. The size of the markers generated varied from 250 bp in OPF 16 to 2100 bp in OPA 10. With eight of the 15 primers used in the study there were differences in the markers generated in the controls and in the recurrently selected lines of either or both cultivars. There were no differences in the

markers produced by the leaves and the unchallenged control with any of the 15 primers. The results of the amplifications with various primers are presented in Table 6.2.

6.3.1 'Carabao'

Among the 15 primers tried, the selected putatively tolerant lines showed variation in markers with 8 primers. Thirteen markers that were present in the controls, were not present in one or both selected putatively tolerant lines. Most of the variation was with PRL III, accounting for 11 out of the 13 markers that were absent. Only 2 primers, OPA 10 and OPA 20, caused deletions in markers with PRL V. There were 7 additional markers, caused by 4 primers, that were present in the selected putatively tolerant lines. Of these, 5 were in PRL III. While none of the OPA primers generated additionals markers with PRL V, OPF 7 and OPF 13 generated markers of 1200 and 1100 bp, respectively (Table 6.2.). A 800 bp marker was strongly expressed by PRL III with OPA 19, while this marker was present only very faintly in the controls and PRL III .

6.3.2 'Hindi'

Only 5 out of the 15 primers tried caused variation in marker generation. Eleven markers that were present in the controls were absent in one or both of the recurrently selected resistant lines. There were 4 additional markers in the recurrently selected resistant lines, of which 3 were in PRL V (Table 6.2.).

Table 6.2. RAPD generated markers with mango leaves and proembryonic masses.

		'CARABAO'				'HINDI'			
Primer	BP	L	C	PRL III	PRL V	L	C	PRL III	PRL V
OPA 7	1300	1	1	1	1	1	1	1	1
	800	1	1	1	1	1	1	1	1
	700	1	1	1	1	0	0	0	0
OPA 8	2072	1	1	1	1	1	1	1	1
OPA 10	2100	1	1	1	1	1	1	1	0
	1500	1	1	0	1	0	0	0	1
	1150	1	1	1	1	1	1	1	1
	800	0	0	1	0	1	1	1	0
	700	0	0	1	0	1	1	1	0
	625	1	1	1	0	1	1	1	1
	500	1	1	1	1	1	1	1	1
OPA 11	1350	1	1	1	1	1	1	1	1
	1200	1	1	0	1	0	0	0	0
	1100	1	1	1	1	1	1	1	1
	1000	1	1	0	1	1	1	1	1
	825	1	1	1	1	1	1	1	1
	650	1	1	1	1	1	1	1	1
	475	1	1	1	1	1	1	1	1
	400	1	1	0	1	1	1	0	0
OPA 13	1300	1	1	1	1	1	1	1	1
	1100	1	1	0	1	0	0	1	1
	1000	0	0	1	0	1	1	0	0
	775	1	1	0	1	1	1	1	1
	700	0	0	1	0	1	1	0	0
	650	1	1	1	1	1	1	1	1
	550	1	1	1	1	1	1	1	1
OPA 18	1350	1	1	1	1	1	1	1	1
	1000	1	1	1	1	1	1	1	1

cont. on next page

OPA 19	1500	1	1	1	1	1	1	1	1
	1350	1	1	1	1	1	1	1	1
	850	1	1	1	1	1	1	1	1
	500	1	1	1	1	1	1	1	1
	375	1	1	0	1	0	0	0	0
OPA 20	1500	1	1	1	1	1	1	1	1
	1000	1	1	1	0	1	1	1	1
	675	1	1	1	1	1	1	1	1
	550	1	1	0	1	1	1	0	0
OPF 6	2072	1	1	1	1	1	1	1	1
	1150	1	1	0	1	1	1	1	1
	900	1	1	1	1	1	1	1	1
	600	1	1	1	1	1	1	1	1
	500	1	1	1	1	1	1	1	1
OPF 7	1400	1	1	1	1	1	1	1	1
	1300	1	1	1	1	1	1	1	1
	1200	0	0	1	1	1	1	0	0
	950	1	1	0	1	0	0	0	1
	650	1	1	1	1	1	1	1	1
	600	1	1	0	1	0	0	0	1
OPF 12	1400	1	1	1	1	1	1	1	1
	300	1	1	1	1	1	1	1	1
OPF 13	1500	1	1	1	1	1	1	1	1
	1150	1	1	1	1	1	1	1	1
	1100	0	0	0	1	0	0	0	0
OPF 15	1350	1	1	1	1	1	1	1	1
	1100	1	1	1	1	1	1	1	1

cont. on next page

OPF 16	900	1	1	1	1	1	1	1	1
	750	1	1	1	1	1	1	1	1
	650	1	1	1	1	1	1	1	1
	550	1	1	1	1	1	1	1	1
	350	1	1	1	1	1	1	1	1
	250	1	1	1	1	1	1	1	1
OPF 19	750	1	1	1	1	1	1	1	1
	600	1	1	1	1	1	1	1	1

Legend
L - Leaf
C - Unchallenged control
PRL III - Putatively Resistant Line III
PRL V - Putatively Resistant Line V

Together the variation uncovered by these 8 primers revealed 11 additional bands in either of the recurrently selected lines in one or both cultivars. On the other hand, 24 markers that were present in the controls were absent in one or more resistant lines. Of the 11 additional markers generated, 10 were present in the controls of the other cultivar. For instance, the 700 and 800 bp markers found only in PRL III of 'Carabao' with primer OPA 10 is also present in the controls of 'Hindi.' Only one 1100 bp marker was uniquely produced by PRL V of 'Carabao.'

6.4 Discussion

One of the major consequences of maintaining long term suspension cultures is the accumulation of mutant cell types, thereby altering the theoretical genetic uniformity of the cultures. Culture regimes and medium components, especially

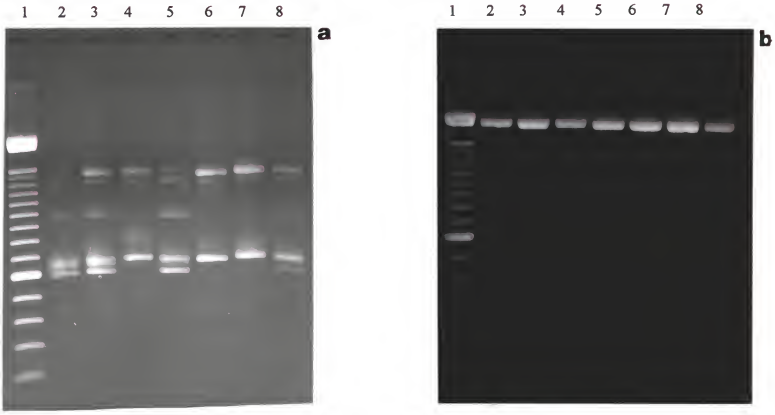

Fig. 6.1. Amplified products with primer (a) OPA 7 and (b) OPA 8. [Lanes. 1. 100 bp marker, 2. 'Carabao' leaf, 3. 'Carabao' control, 4. 'Carabao' PRL III, 5. 'Carabao' PRL V, 6.'Hindi' leaf, 7. 'Hindi' control, 8. 'Hindi' PRL III]

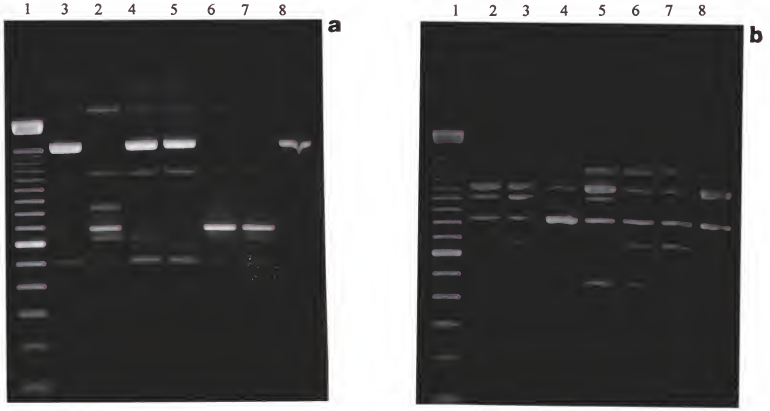

Fig. 6.2. Amplified products with primer (a) OPA 10 and (b) OPA 11. [Lanes. 1. 100 bp marker, 2. 'Carabao' leaf, 3. 'Carabao' control, 4. 'Carabao' PRL III, 5. 'Carabao' PRL V, 6.'Hindi' leaf, 7. 'Hindi' control, 8. 'Hindi' PRL III]

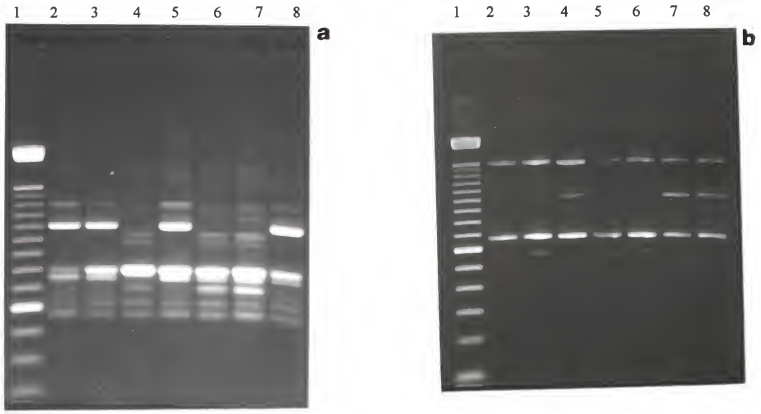

Fig. 6.3. Amplified products with primer (a) OPA 13 and (b) OPA 20. [Lanes. 1. 100 bp marker, 2. 'Carabao' leaf, 3. 'Carabao' control, 4. 'Carabao' PRL III, 5. 'Carabao' PRL V, 6.'Hindi' leaf, 7. 'Hindi' control, 8. 'Hindi' PRL III]

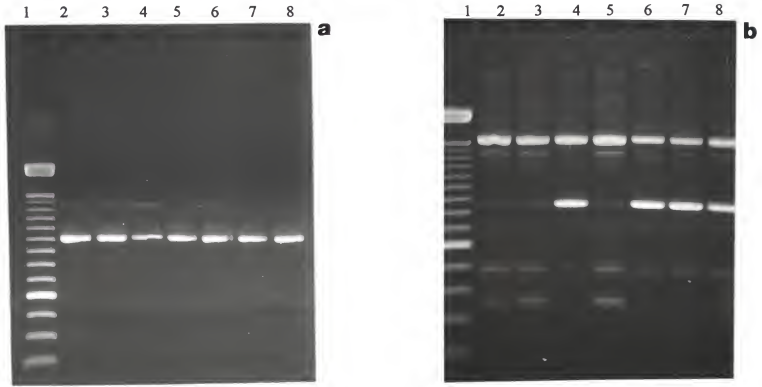

Fig. 6.4. Amplified products with primer (a) OPA 18 and (b) OPA 19. [Lanes. 1. 100 bp marker, 2. 'Carabao' leaf, 3. 'Carabao' control, 4. 'Carabao' PRL III, 5. 'Carabao' PRL V, 6.'Hindi' leaf, 7. 'Hindi' control, 8. 'Hindi' PRL III]

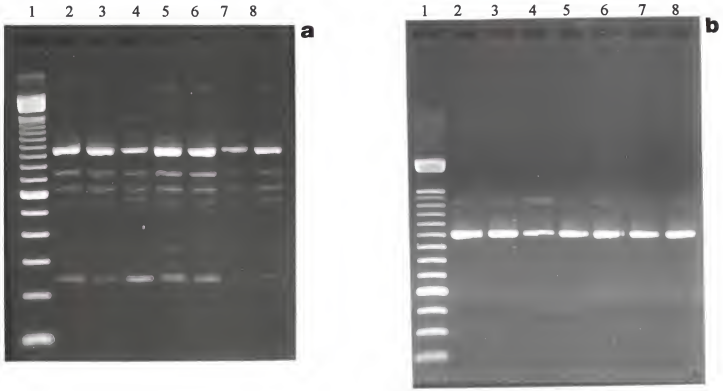

Fig. 6.5. Amplified products with primer (a) OPF 16 and (b) OPA 12. [Lanes. 1. 100 bp marker, 2. 'Carabao' leaf, 3. 'Carabao' control, 4. 'Carabao' PRL III, 5. 'Carabao' PRL V, 6.'Hindi' leaf, 7. 'Hindi' control, 8. 'Hindi' PRL III]

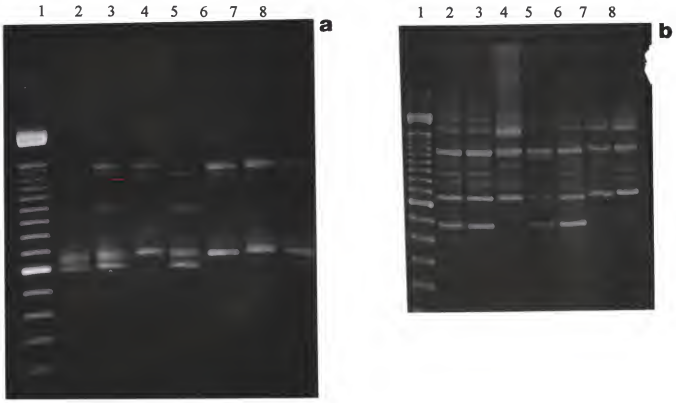

Fig. 6.6. Amplified products with primer (a) OPF 7 and (b) OPF 13. [Lanes. 1. 100 bp marker, 2. 'Carabao' leaf, 3. 'Carabao' control, 4. 'Carabao' PRL III, 5. 'Carabao' PRL V, 6.'Hindi' leaf, 7. 'Hindi' control, 8. 'Hindi' PRL III]

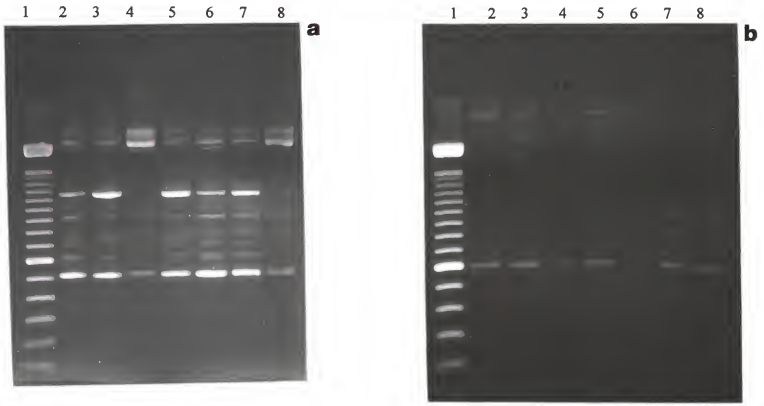

Fig. 6.7. Amplified products with primer (a) OPF 19 and (b) OPF 6. [Lanes. 1. 100 bp marker, 2. 'Carabao' leaf, 3. 'Carabao' control, 4. 'Carabao' PRL III, 5. 'Carabao' PRL V, 6.'Hindi' leaf, 7. 'Hindi' control, 8. 'Hindi' PRL III]

growth regulators are often cited as being important causes of this variation (Bayliss, 1977; 1980). Embryogenic suspension cultures are subjected to a gradual depletion of nutrients, and upon subculture into a fresh medium, they are exposed to elevated levels of nutrients and growth regulators. This cycle is repeated several times with mango during culture proliferation. Such prolonged maintenance in liquid cultures alters the intracellular deoxyribonucleotide (dNTP) composition, resulting in nucleotide pool imbalance (Lee and Phillips, 1988). Imbalances within the nucleotide pool have been associated with serious genetic consequences including DNA replication, repair, degradation (Haynes, 1985), extranuclear mutations, mitotic recombinations and chromosomal aberrations (Kunz, 1982; Kunz and Haynes, 1982). Absence of variability in RAPD markers generated by 15 different primers between leaves and unchallenged proembryonic masses indicates: 1) the proembryonic masses are of nucellar origin and hence are maternal and 2) proembryonic masses leaves indicate and 3) that do not undergo any detectable genetic changes in suspension culture in the absence of selection with a phytotoxic metabolite produced by *C. gloeosporioides*. The changes in RAPD markers generated by the selected lines PRL III and PRL V must therefore be the result of exposure to the putatively phytotoxic metabolite or selection pressure. Therefore, we are comparing isogenic or near isogenic lines (NILs) of each cultivar, which differ only in their ability to mutate during *in vitro* selection against culture filtrate. Any polymorphism that exists between the recurrently selected lines and controls should be associated with the selection process. This is essentially comparable to bulked segregation analysis described by Michelmore *et al.* (1991) and which is often used with herbaceous species.

During *in vitro* selection, the culture environment imposes an enormous stress on growing embryogenic cultures. Such an environmental stress has been observed to cause changes in DNA sequence copy number in maize (*Zea mays* L.) (Phillips, 1978; Rivin *et al.*, 1986) and flax (*Linum usitatissimum* L.) (Cullis, 1983; Cullis and Cleary, 1985). Similarly, gene amplification in response to *in vitro* selection for herbicide tolerance has been reported in alfalfa (*Medicago sativa* L.) (Donn *et al.*, 1984) and petunia (*Petunia hybrida* L.) (Shah *et al.*, 1986). Activation of transposable elements in maize regenerants following *in vitro* regeneration has been reported (Peterson, 1986; Peschke *et al.*, 1987) and transposon-like activity has been detected in alfalfa tissue cultures (Groose and Bingham, 1986a,b). In their historical review, Larkin and Scowcroft (1981) have listed other genetic changes, such as chromosome breakage, mitotic recombination and late replication of heterochromatin following *in vitro* regeneration, as possible sources of somaclonal variation. In the present study, generation of additional RAPD markers could have resulted from such chromosomal rearrangements altering the DNA sequence, following *in vitro* selection; however, absence of markers in the selected lines was most frequently observed. This suggests the probable occurrence of deletion mutation(s) within the DNA during recurrent selection. Deletion of one or more bases in the genomic DNA can lead to the loss of a priming site for the primer, and therefore the absence of marker(s) (Michelmore *et al.*, 1991). Deletions and single base changes are common types of polymorphisms detected by RAPDs (Rafalsky and Tingey, 1993). Moreover, RAPDs have also been found to be useful for identification of somatic mutations among clonal derivatives (Collins and Symons, 1993).

In an earlier study (Chapter 5), increased activity of chitinase and β-1,3-glucanase was observed in the recurrently selected, putatively resistant embryogenic lines of both mango cultivars. Several proteins are normally induced by plants in response to a pathogen infection (Roby *et al.*, 1990; Graham and Sticklen, 1994). Chitinases and β-1,3-glucanases are elicited in a non-specific manner (Boller *et al.*, 1983; Lamb *et al.*, 1992) and are the most widely recognized pathogenesis-related proteins (Legrand *et al.*, 1987). However, it remains to be seen whether the polymorphism exhibited by the selected lines could be related to these defense proteins. One possibility is that the deletions in the DNA sequence in the selected lines might have occurred in the promotor region of these defense genes leading to the activation of the promotor. In this scenario, there would be increased constitutive expression of these defense proteins in the selected lines. Such activation of a chitinase promotor due to pathogen infection has been reported in transgenic tobacco plants (Roby *et al.*, 1990).

The amplified products generated by RAPDs can be useful for constructing genetic linkage maps (Williams *et al.*, 1990; Ronning, 1995) or tagging chromosomes (Martin *et al.*, 1991). These are extremely useful for the genetic improvement of perennial species, in which conventional breeding is severely handicapped. Molecular markers help in genotype selection at the cellular or tissue level (Tanksley *et al.*, 1989) saving time and increasing efficiency. Marker aided selection (MAS) (Tulseiram *et al.*, 1992) effectively shortens the evaluation cycle of woody perennials (Strauss *et al.*, 1994). It might be possible to identify and clone anthracnose resistant genes from the polymorphic markers generated in this study. With successful demonstration of genetic transformation in mango (Mathews *et al.*,

1992), identification and cloning of anthracnose resistant gene(s) offers great potential for developing anthracnose-resistant mango cultivars.

CHAPTER 7
SOMATIC EMBRYOGENESIS

7.1 Introduction

PEM from the recurrently selected lines were multiplied in suspension culture for approximately 12 weeks. After proliferating sufficient quantities of PEM, the nature of resistance to the selection pressure was characterized as described in Chapters 3-6. Concurrently, somatic embryogenesis was initiated using part of the proliferated PEM.

Mango seeds have extremely long maturation periods, exceeding 120 days (DeWald *et al.*, 1989a), probably due to the development of massive cotyledons. A similar lengthy period is encountered for somatic embryo maturation. Such a long maturation period warrants manipulations of culture media at key steps, especially to prevent hyperhydricity and precocious germination, two of the most crucial factors in mango somatic embryo development (Litz *et al.*, 1993; 1994). In addition, efficient recovery of mango somatic embryos could be used as a model for regenerating other tropical tree species having large embryos (DeWald *et al.*, 1989b).

Difficulties in regeneration following *in vitro* selection have been reported in peach (Hammerschlsg, 1988) and maize (Wolf and Earle, 1990). The current study aimed at 1) examining if *in vitro* selection using culture filtrate or partially purified phytotoxic metabolite from *C. gloeosporioides* has altered somatic embryogenesis in

phytotoxic metabolite from *C. gloeosporioides* has altered somatic embryogenesis in mango and 2) to determine if somatic embryogenesis in mango is genotype dependent.

7.2 Materials and Methods

Initiation and maturation of 'Hindi' somatic embryos. Embryogenic cultures were established on semi-solid medium consisting of B5 major salts (Gamborg *et al.*, 1968), MS minor salts and organics (Murashige and Skoog, 1962), 400 mg liter^{-1} glutamine and 1 mg liter^{-1} 2,4-D (DeWald *et al.*, 1989a) and were grown at room temperature (25-27°C) in darkness. Proembryonic masses (PEM) were maintained in liquid growth medium of the same formulation at 120 rpm in semi darkness at room temperature and were subcultured at weekly intervals into fresh growth medium. Somatic embryo maturation was induced by subculturing embryogenic PEM into liquid growth medium without 2,4-D, but containing 50 µg liter $^{-1}$ benzyladenine (Litz *et al.*, 1993). The ammoniacal form of nitrogen was deleted. This formulation is hereafter referred to as LEM (liquid embryogenesis medium). Actively proliferating suspension cultures were sieved through a 100 µm nylon mesh (Monsalud, 1994) and the fine fraction was recovered in LEM. Approximately 1.0 g of the fine PEM were cultured in a 125 ml Erlenmeyer flask containing 50 ml of LEM and incubated on a rotary shaker at 120 rpm under diffused light. PEM were subcultured into fresh fresh LEM at weekly interval. In

approximately 6 weeks, late heart to early cotyledonary-shaped somatic embryos started to appear. When most of the somatic embryos in LEM reached the cotyledonary stage, they were sieved through a 3,000 μm nylon mesh screen. The fraction that passed through the sieve was recultured in LEM for further development, while the fraction that was retained on the sieve, consisting of larger somatic embryos, were plated onto semisolid somatic embryogenesis medium (SEM). SEM is the same as LEM, but solidified with Gel-Gro. In order to avoid hyperhydricity in somatic embryos the Gel-Gro concentration was raised to 3.0 gl^{-1} (Monsalud, 1994; Monsalud *et al.*, 1995). In about 3 weeks, opaque somatic embryos (≥ 5mm) started developing in these plates. These opaque somatic embryos were subcultured onto SEM containing 2.0 gl^{-1} Gel-Gro for further development. After 3-4 weeks, the somatic embryos were subcultured onto semisolid maturation medium I (MM I) (Appendix A). The plates were incubated in darkness until germination. Somatic embryos that germinated in MMI were subcultured onto fresh meidum in petri dishes containing MM II (Appendix A). Usually the number of somatic embryos on MM II was limited to a maximum of 3 per plate. The plates were incubated in a growth chamber at 25°C with a 16 h photoperiod. Somatic embryos were subcultured at monthly intervals; however, those somatic embryos that produced shoots were transferred to G 7 culture containers (Sigma) containing MM II medium and incubated in a high CO_2 chamber with a 16 h photoperiod. The CO_2 content (20,000 ppm) in the chamber was maintained by supplying monitored

CO_2 from a gas tank of the same mixture (20,000 ppm CO_2 in nitrogen). The G 7 culture containers were covered with transparent suncaps (Supelco). Plantlets with more than 3 leaves were transferred *ex vitro*.

Somatic Embryo Initiation and Maturation in 'Carabao.' Proliferating PEMs were sieved and subcultured in LEM for 4 weeks, as described for 'Hindi' (see above). After 4 weeks the cultures were sieved through 1000 µm nylon mesh screen and the larger fraction was subcultured on SEM. The plates were incubated in darkness. Proembryonic masses were subcultured at monthly intervals onto fresh SEM. Approximately 6-8 weeks later, white cotyledonary somatic embryos developed (Fig. 7.1). Somatic embryos that were ≥ 5mm were subcultured onto fresh SEM for further development. Fully grown somatic embryos (≥ 20mm) were subcultured onto MM I. Maturation, germination and plantlet production were accomplished as described for 'Hindi'.

In Vitro Grafting. In order to improve plant recovery from mango somatic embryos, attempts were made to graft the plantlets produced *in vitro*, as described for cacao (Aguilar *et al.*, 1993). *In vitro* germinated seedlings, approximately 3 weeks after shoot growth, were decapitated transversely 1 cm above the hypocotyl using a sterile surgical blade under the laminar flow hood. A longitudinal incision of approximately 0.5 cm was made from the cut surface. This formed the rootstock. Only plantlets consisting of a well developed primary root with a few laterals were chosen as rootstocks. An *in vitro* derived shoot from another plantlet of same stem

thickness was used as the scion. The cut end of the scion was shaped like a wedge by giving two sloping cuts in opposite sides of the base of the scion and inserted into incision in the rootstock. The graft joint was covered with semisolid medium (MM II supplemented with 50 ml liter $^{-1}$ of antibiotic mixture described by Young *et al.*, 1984). The grafted plantlets were transferred to a G 7 culture container containing MM II medium and incubated in a growth chamber.

Extraction of extracellular proteins and assay for chitinase produced by mature somatic embryos. Somatic embryos were cultured in semisolid medium (SEM) for four weeks. After subculturing the somatic embryos, the spent medium was diluted with 20 ml of sterile deionized water and stirred overnight using a magnetic stirrer. The resulting suspension was filtered through a sterile kimwipe. Extracellular proteins were precipated using cold ethanol, quantified and assayed for chitinase activity as described in Chapter 5.2, Materials and Methods, pp.59-61.

7.3 Results

'Hindi.' Production of mature somatic embryos of "Hindi' occurred efficiently. More than 300 mature somatic embryos were produced from each of unchallenged control, PRL III and PRL V (Table 7.1). More than 80% of the somatic embryos produced by each line germinated. Somatic embryogenesis could not be achieved with line PTR of either cultivar. Efficiency of somatic embryogenesis decreased with length of culture in LEM. Developmental anomalies

including polycotyly and fasciation were observed in all the lines in approximately the same frequency. More frequently, monocotyly was observed. Occurrence of direct, secondary embryogenesis from the hypocotyl region was also observed. Mature somatic embryos were 2 cm in length; however, the number of somatic embryos that produced shoots and survived conversion as *in vitro* plantlets was very low. Only 27 somatic embryos produced shoots from all three lines. The total time taken for the somatic embryos to germinate after subculture from 2,4-D medium was 24-30 weeks. Most of the plantlets had very weak shoots and scaly leaves.

Table 7.1 Somatic embryo and plantlet production in various lines of 'Hindi' and 'Carabao.'

Cultivar	'HINDI'			'CARABAO'		
Lines	Number of SEs produced	Number of SEs germinated	Number of plantlets produced *in vitro*	Number of SEs produced	Number of SEs germinated	Number of plantlets produced *in vitro*
Control	345	320	9	176	154	7
PTL III	328	306	11	203	181	9
PTL V	276	243	7	155	132	4
TOTAL	949	869	27	534	467	20

'Carabao.' Although the PEM of 'Carabao' proliferated in maintenance medium like 'Hindi,' somatic embryo development did not occur following subcultures in LEM; however, somatic embryos could be induced following subculture onto semisolid medium. Formation of cotyledonary somatic embryos required 12-14 weeks in contrast with 'Hindi' (5-6 weeks). Thereafter, the development occurred much more rapidly. Although the number of somatic embryos produced by 'Carabao' was much lower in comparison with 'Hindi' (Table 7.1), the somatic embryos were larger and more normal in appearance (Fig. 7.1). Fully mature 'Carabao' somatic embryos were as long as 3 cm. Developmental anomalies included absence of bipolarity, polycotyly and fasciation. Secondary embryogenesis was frequently observed in 'Carabao' in comparison with 'Hindi.' In contrast with 'Hindi,' shoot development occurred when the somatic embryos were still on MM I in darkness. No 'Carabao' somatic embryos produced shoots after being transferred to light; however, somatic embryos that produced shoots in darkness, grew very rapidly after transfer to light. A total of 20 plantlets were produced over 12 months in all the 3 lines. Although the plantlets appeared to be healthier than 'Hindi,' with fully expanded leaves *in vitro* (Fig. 7.2), they also have been difficult to establish in soil.

In vitro grafting of 'Carabao' was not successful; however, only 10 grafts were tried. Of the 10 grafts, 9 died as a result of microbial contamination. One graft

remained green for over 3 months; however, it did not produce any new shoot growth.

Assay for chitinase activity. Chitinase isozymes were visualized in the overlay gels following native PAGE. In 'Hindi,' all the three lines- control, PRL III and PRL V- exhibited three isozymes of 30, 32 and 45 kD; however, the 45 kD isozyme was very weak in the control in comparison with the resistant lines. In 'Carabao,' only two isozymes were clearly visible. The 45 kD isozyme was much strongly exhibited by the resistant lines.

Fig. 7.1. A germinating 'Carabao' somatic embryo.

Fig. 7.2. An *in vitro* derived 'Carabao' plantlet.

CHAPTER 8
SUMMARY AND CONCLUSIONS

The studies revealed the *in vitro* production of phytotoxic metabolites by the fungus, *C. gloeosporioides*. Partial purification of the phytotoxic metabolite from the culture filtrate was achieved. The phytotoxicity of this metabolite was demonstrated to be of non specific nature, since it inhibited the germination of two nonhosts, lettuce and tobacco. In addition, the fraction also caused necrotic, anthracnose-like symptoms on mango leaves *in vivo*.

A scheme for recurrent *in vitro* selection of embryogenic cultures of mango was devised. Putatively resistant lines of mango cultivars 'Hindi' and 'Carabao' were established by recurrently selecting embryogenic suspension cultures with progressively increasing concentrations of culture filtrate, with a constant concentration of the culture filtrate and with the phytotoxic metabolite. The growth of embryogenic cultures was significantly inhibited during recurrent selection in comparison with the unchallenged and medium controls. The resistant lines significantly inhibited the mycelial growth of *C. gloeosporioides* in dual culture in comparison with the unchallenged controls of both cultivars. Conditioned medium studies containing 20% (v/v) macerated cultures from the selected lines did not inhibit mycelial growth, thereby indicating that the antifungal compounds produced by the selected lines are extracellular.

Analysis of the spent medium of the various resistant lines and of the control of the two cultivars revealed significant differences in the secretion of extracellular proteins. SDS-PAGE separation of the extracellular proteins revealed differences in the protein profile between the cultivars. In addition, differences between the unselected and selected lines were also evident. The resistant lines of both cultivars exhibited a new peptide of approximately 45 kD that co-migrated with a peptide from a chitinase standard. Assay for chitinase using nondenatured PAGE followed by overlay gel digestion revealed a new chitinase isozyme being exhibited by all the resistant lines of 'Hindi', while there was enhanced expression of two other isozymes in both 'Hindi' and 'Carabao'. Increased expression of β-1,3-glucanase was also observed in the resistant lines of both cultivars in comparison with the controls. There was no visible difference between the resistant lines and controls with respect to total soluble proteins extracted from the embryogenic callus or from the mature somatic embryos.

Analysis of the genomic DNA of the resistant lines, controls and leaves from the parent trees (from which the nucellar cultures were established) using RAPD revealed differences between the resistant lines and the controls. Most of the differences involve absence of markers in the resistant lines, indicating some deletion mutations have occurred in the selected lines during *in vitro* selection. In addition, there were no differences between the leaf DNA and the unchallenged controls indicating 1) the cultures are of maternal origin and 2) no genetic change has

occurred in the embryogenic cultures due to prolonged maintenance in suspension culture. It is hypothesised that the differences observed between the unchallenged controls and the resistant, near isogenic lines can serve as markers to identify the anthracnose resistance genes in mango.

Cultivar differences were observed between 'Hindi' and 'Carabao' in somatic embryogenesis indicating that somatic embryogenesis is genotype dependent. *In vitro* grafting of mango was unsuccessful. Identification and cloning the anthracnose resistance gene and genetically transforming mango using established protocols could enhance anthracnose tolerance in mango.

APPENDIX A

MEDIA FORMULATION FOR MANGO NUCELLAR TISSUE CULTURE

1. $B5^{+}$ (Gamborg *et al.*, 1968) (10X) 1 liter solution

KNO_3	25g
$CaCl_2.2H_2O$	1.5 g
$(NH_4)_2SO_4$	1.34 g
$MgSO_4.7H_2O$	2.5 g
$NaH_2PO_4.H_2O$	1.5 g

2. $B5^{-}$ (Gamborg *et al.*, 1968) (10X) 1 liter solution

KNO_3	25 g
$CaCl_2.2H_2O$	1.5 g
$MgSO_4.7H_2O$	2.5 g
$NaH_2PO_4.H_2O$	1.5 g

3. Murashige & Skoog (1962) Organic (MSO) Compounds (10X) 1 liter solution

Myo-inositol	10 g
Nicotinic Acid	50 mg
Pyridoxine HCl	50 mg
Thiamine HCl	10 mg
Glycine	200 mg

4. Murashige & Skoog (1962) Minor Elements (10X) 1 liter solution

H_3BO_3	6.2 mg
$CoCl_2.6H_2O$	0.025 mg
$CuSo_4$	0.025 mg
Na_2-EDTA	37.3 mg
$FeSO_4.7H_2O$	27.8 mg
$MnSO_4.H_2O$	16.9 mg
$MoO_4Na_2.2H_2O$	0.25 mg
KI	0.83 mg
$ZnSO_4.7H_2O$	8.6 mg

5. Proliferation Medium

$B5^+$ (10X)	100 ml
MS organics (10X)	10 ml
MS minors (10X)	10 ml
Sucrose	60 g
Glutamine	400 mg
2,4-D	1 mg

6. LEM

$B5^-$ (10X)	100 ml
MS organics (10X)	10 ml
MS minors (10X)	10 ml
Sucrose	60 g
Glutamine	400 mg
BA	50µg

7. MM I

$B5^-$ (10X)	100 ml
MS organics (10 X)	10 ml
MS minors (10X)	10 ml
Sucrose	40 g
Glutamine	400 mg
Activated charcoal	30 mg
Gel Gro	2 g
Coconut H_2O	200 ml *

8. MM II

B5 (10X)	50 ml
MS organics (10X)	10 ml
MS minors (10X)	10 ml
Sucrose	20 g
Glutamine	400 mg
Activated charcoal	30 mg
Gel Gro	2 g
Coconut Water	200 ml *

* Filter-sterilized

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BIOGRAPHICAL SKETCH

Jayasankar Subramanian was born in Lalgudi, a small village in Tamilnadu, India. He received B.Sc. (Hort.) and M.Sc. (Hort.) from the Tamilnadu Agricultural University, Coimbatore, India in 1983 and 1986, respectively. In 1983, he won the first rank in the Indian Council of Agricultural Research junior fellowship national competitive examination in horticulture, with which he did his masters program. Since April, 1986, he has been working as an Assistant Professor of Horticulture in the same school.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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